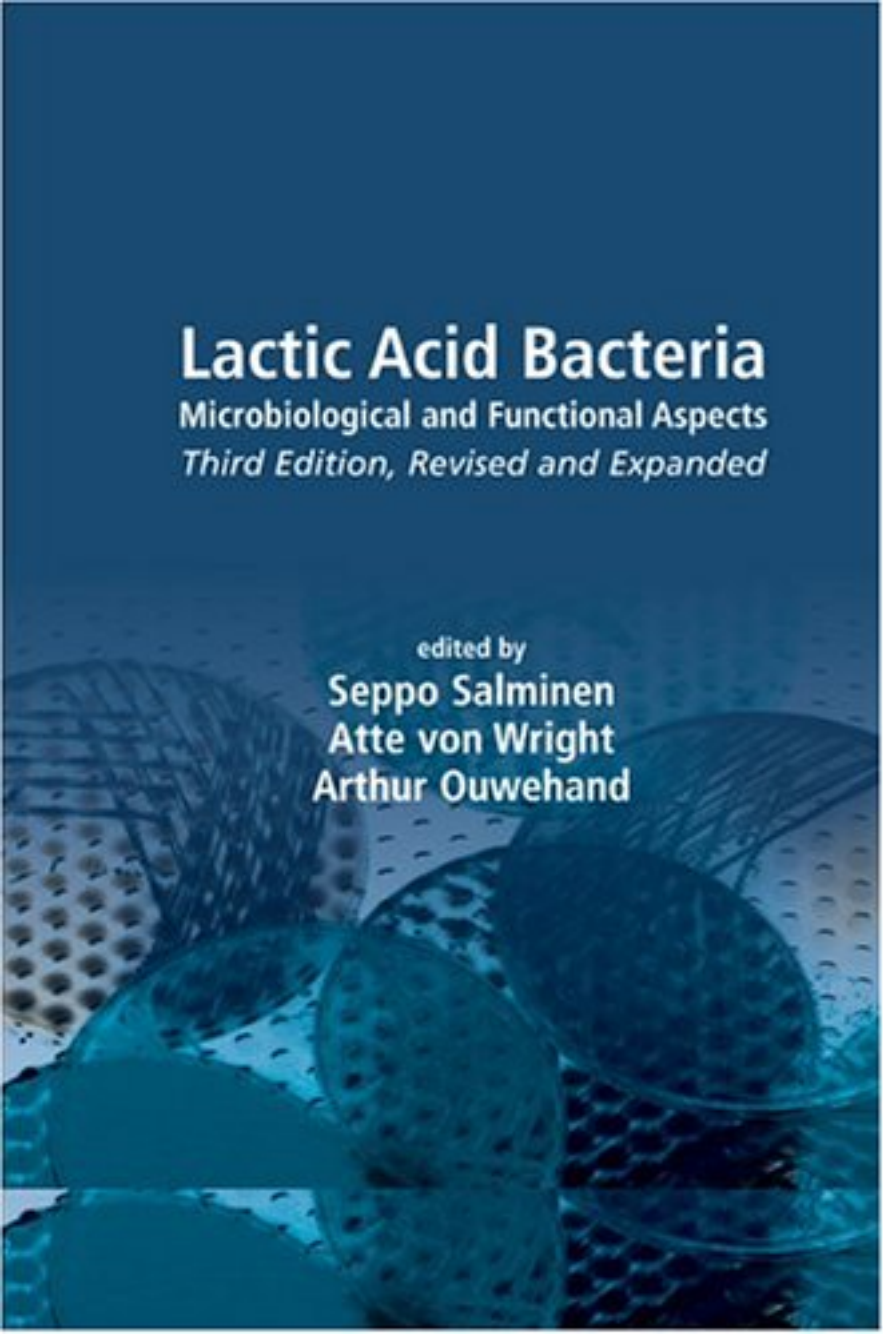
Lactic Acid Bacteria

Microbiological and Functional Aspects

Third Edition, Revised and Expanded

edited by
Seppo Salminen
Atte von Wright
Arthur Ouwehand



Lactic Acid Bacteria

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Preface to the Third Edition

This third edition of *Lactic Acid Bacteria* covers the progress in this field of research over the past five years. The pace of development, already impressive when the second edition was being compiled, has shown no signs of slowing down. Consequently, most chapters in this new edition have been completely rewritten, several new contributors have been recruited, and some totally new chapters have been included, covering topics such as mathematical modeling, vegetable fermentation, methods for analysis of the gut microbiota, and probiotics for fish. It has been more and more of a challenge to keep the volume comprehensive, up-to-date, and concise. While it remains up to the reader to judge how well these goals have been achieved, we feel that the present volume gives a valuable overview of the present status of this rapidly expanding interdisciplinary area of research. As in the previous editions, a special emphasis has been placed on the health aspects of lactic acid bacteria, although, as can be seen in the table of contents, other relevant applications have also been covered. The intended audience includes, among others, microbiologists, food technologists, nutritionists, clinicians, product development experts, and regulatory experts.

Seppo Salminen
Atte von Wright
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Preface to the Second Edition

The first edition of *Lactic Acid Bacteria* was a profound success and well received. This, together with the very rapid progress in the field of research on lactic acid bacteria, has created a need for the second edition of the volume sooner than we had thought. Most of the material has been completely rewritten, and some totally new chapters have been included, although few changes have been made in some technical chapters.

Understanding of the scientific basis of probiotic research, which was anticipated in the previous edition, has advanced in great strides. Consequently, new data on immunology, animal probiotics, and the role of propionic acid bacteria have been added. The importance of bacteriophages, both as a practical problem and as a molecular biological tool, has merited a special chapter. Other chapters have been updated for the most recent research findings and regulatory developments.

We feel that this book provides the reader with a concise overview of a rapidly progressing field. Thus, it is an invaluable aid in guiding the reader through the web of accumulating new research data and in summarizing the fragmentary information available in specialist publications. Also, recent rapid developments in the area of functional foods should make the present edition valuable to an even wider audience.

Seppo Salminen
Atte von Wright

Preface to the First Edition

Lactic acid–producing fermentation is an old invention. Many different cultures in various parts of the world have used it to improve the storage qualities, palatability, and nutritive value of perishable foods such as milk, vegetables, meat, fish, legumes, and cereals. The organisms that produce this type of fermentation, lactic acid bacteria, have had an important role in preserving foods, preventing food poisoning, and indirectly feeding the hungry on every continent.

In the developed world, lactic acid bacteria are mainly associated with fermented dairy products such as cheese, buttermilk, and yogurt. The use of dairy starter cultures has become an industry during this century. Because of this, the technological aspects of lactic acid fermentation have been well covered in both research and training in food sciences.

Since the days of the Russian scientist Metchnikoff, lactic acid bacteria have also been associated with beneficial health effects. Today an increasing number of health foods and so-called functional foods as well as pharmaceutical preparations are promoted with health claims based on the characteristics of certain strains of lactic acid bacteria. Most of these strains, however, have not been thoroughly studied, and consequently the claims are not well substantiated. Moreover, the accepted standards of clinical protocols, including double-blind randomized study designs, have not been applied in most "health-claim" studies—health benefits are judged mainly using subjective criteria. Additionally, the specific bacterial strains used in the studies are often poorly identified. Most information about the health effects of lactic acid bacteria is thus anecdotal. There is a clear need for critical study of the effects on health of strain selection and the quality of fermented foods and their ingredients. Clinical studies should be properly conducted as double-blind, placebo-controlled randomized trials. Both the defined bacterial strains and the proposed products should be studied to verify results. Only such studies produce the solid data that can back up health claims.

This book reviews current developments in the study of lactic acid bacteria using the above-mentioned criteria. An overview of the taxonomy and general physiology of lactic acid bacteria is given. A discussion of the genetics of lactic acid bacteria as a future area of interest is included as well as a chapter on the technological aspects of manufacturing functional lactic acid bacteria starters. Many chapters consider our present

knowledge of the effects of lactic acid bacteria in human health and disease and as animal probiotics.

One chapter of particular interest describes the development of individual lactic acid microflora. It was written by an Estonian research group that worked in association with the former Soviet space program. These results have not been previously published in the West.

Thus, this book attempts to shed light on little-known and controversial aspects of lactic acid bacteria and their applications. As new techniques as well as new interest in these organisms develop, the anecdotal evidence on the health benefits of specific strains of lactic acid bacteria is slowly being replaced by a more scientific outlook. This book should serve as an important introduction to any student or scientist interested in these developments.

In particular, those working with lactic acid bacteria and fermented foods or feed products within universities and the food industry should find this book most interesting. It will also be helpful to dairy scientists and technologists, both as a textbook and as a handbook for product development. It will be useful to government organizations developing regulatory policies for products based on lactic acid fermentation and bacteria, especially when health claims are concerned. Finally, consumer groups interested in the effects of lactic acid bacteria may benefit from the comprehensive reviews in this volume.

Readers are referred to most of the recent literature in the area, covering the subject well from various aspects. Outr aim has been to give an overview of a rapidly changing and extremely important area of food and nutrition research.

Seppo Salminen
Atte von Wright

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Lactic Acid Bacteria: Classification and Physiology

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I. SUMMARY

Lactic acid bacteria (LAB) constitute a group of gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the group is gram-positive, nonsporing, nonrespiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. The LAB term is intimately associated with bacteria involved in food and feed fermentation, including related bacteria normally associated with the (healthy) mucosal surfaces of humans and animals. The boundaries of the group have been subject to some controversy, but historically the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* form the core of the group. Taxonomic revisions of these genera and the description of new genera mean that LAB could, in their broad physiological definition, comprise around 20 genera. However, from a practical, food-technology point of view, the following genera are considered the principal LAB: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella.* The genus *Bifidobacterium*, often considered in the same context as the genuine lactic acid bacteria and sharing some of their typical features, is phylogenetically unrelated and has a unique mode of sugar fermentation. The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. Chemotaxonomic markers such as fatty acid composition and constituents of the cell wall are also used in classification. In addition, the present taxonomy relies partly on true phylogenetic relationships,

which have been revealed by extensive work on determining rRNA sequences. Some of the newly described genera are most easily determined with oligonucleotide probes, polymerase chain reaction (PCR)–based technologies using these sequences, or direct sequencing of the 16S rRNA gene. Most genera in the group form phylogenetically distinct groups, but for some, in particular *Lactobacillus* and *Pediococcus*, the phylogenetic clusters do not correlate with the current classification based on phenotypic characters. New tools for classification and identification of LAB are currently replacing and/or complementing the traditional phenotype-based methodologies. The most promising for routine use are 16S rRNA gene sequencing, PCR-based fingerprinting techniques and soluble protein patterns.

Two main sugar fermentation pathways can be distinguished among lactic acid bacteria. Glycolysis (Embden-Meyerhof-Parnas pathway) results almost exclusively in lactic acid as the end product under standard conditions, and the metabolism is referred to as homolactic fermentation. The 6-phosphogluconate/phosphoketolase pathway results in significant amounts of other end products such as ethanol, acetate, and CO_2 in addition to lactic acid, and the metabolism is referred to as heterolactic fermentation. Various growth conditions may significantly alter the end product formation by some lactic acid bacteria. These changes can be attributed to an altered pyruvate metabolism and/or the use of external electron acceptors such as oxygen, in both a respiratory and a non-respiratory mode, or organic compounds.

Lactic acid bacteria create a proton-motive force mainly by means of a membrane-located H^+-ATPase at the expense of ATP. The proton-motive force drives the uphill transport of metabolites and ions into the cell. End-product efflux and electrogenic transport of certain compounds may contribute to the formation of a proton-motive force, thus sparing ATP. Sugar transport is mediated mainly by proton-motive force–dependant permease systems or phosphoenolpyruvate : sugar phosphotransferase systems. Certain components of the phosphoenolpyruvate : sugar phosphotransferase system appear to hold key positions in the global regulation of the sugar metabolism in general, beyond any function in sugar transport. Transport of amino acids and other nutrients is generally mediated by proton-motive force–dependent systems, antiport systems, or ATP-driven systems.

II. GENERAL INTRODUCTION

What is a lactic acid bacterium? Asking this question of scientists in the field would probably result in a fairly uniform answer. This is more because of a historic tradition, rather than the existance of an unequivocal definition of the term. The historic tradition goes back to before the turn of the twentieth century. The term lactic acid bacteria was then used to mean "milk-souring organisms." Significantly, the first pure culture of a bacterium was "*Bacterium lactis*" (probably *Lactococcus lactis*), obtained by J. Lister in 1873. Important progress in the classification of these bacteria was made when the similarity between milk-souring bacteria and other lactic acid–producing bacteria from other sources was recognized[1,2] However, confusion was still prevalent when the monograph of Orla-Jensen[3] appeared. This work had a large impact on the systematics of LAB and, although revised to a considerable extent, the classification basis remains remarkably unchanged. Orla-Jensen used the following characteristics as a basis for classification: morphology (cocci or rods, tetrad formation), mode of glucose fermentation (homo- or heterofermentation), growth at certain "cardinal" temperatures (e.g., 10°C and 45°C), and range of sugar utilization. As will be seen later in this chapter, these characteristics

are still very important in the classification of LAB. After the work of Orla-Jensen, the view emerged that the LAB comprised four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. There has always been controversy as to the boundaries of the group,[4,5] but that subject will not be dealt with here. The classification section of this chapter will concentrate on what historically constituted these four genera. Since 1985 many new genera have been described, most comprising strains previously included in one of the four mentioned above.

Orla-Jensen regarded LAB as a "great natural group," indicating a belief that the bacteria included were phylogenetically related and separate from other groups. At that time, only phenotypic characters could be examined and evaluated as phylogenetic markers. Today we have the means to examine, in detail, macromolecules of the cell, believed to be more accurate in defining relationships and phylogenetic positions. These are, of course, the nucleic acids. Fortunately, nature has provided us with different kinds of nucleic acids for different types of taxonomic studies. Close relationships (at species and subspecies levels) can be determined with DNA-DNA homology studies,[6] and this method is still used in defining what constitutes a species in the prokaryotic world.[7] For determining phylogenetic positions of species and genera, ribosomal RNA (rRNA) is more suitable, since the sequence contains both well-conserved and less conserved regions. It is now a relatively easy task to determine the sequence of rRNA from bacteria. Initially this was done with the reverse transcriptase technique,[8] but it is now being done by sequencing the corresponding genes using polymerase chain reaction (PCR) technology.[9] Comparisons of these sequences provide the most powerful and accurate technique for determining phylogenetic relationships of microorganisms.[10] With this technique, a more clear picture of the phylogeny of LAB has become evident. In addition, rRNA sequencing is an important aid in the classification of LAB, as exemplified by the descriptions of new genera.[11–13] The classification section of this chapter will deal with both the "classical" classification schemes and the current phylogenetic status of LAB.

The physiology of LAB has been of interest ever since it was recognized that these bacteria are involved in the acidification of food and feed products. Increased knowledge of LAB physiology, such as metabolism, nutrient utilization, etc., has been one way to achieve more controlled processes. Today, modern genetic techniques are considered to be promising in this regard. However, efforts in this direction may not be fruitful unless there is a sound understanding of the physiology of these bacteria. The attempts at metabolic engineering of *Lactococcus lactis*[14–17] would not have been possible without understanding of the physiology and biochemistry of this organism. In the era of genomics, where some LAB genomes already have been published[18,19] and many more are underway,[20] we will have access to a wealth of genetic data with enormous potential to improve our understanding of these bacteria. Still, such data are meaningless without their elucidation in a physiological context. The fermentative nature of LAB is also of considerable academic interest, since it makes them excellent model systems for the study of energy transduction, solute transport, and membrane biology.[21–26]

The designation "*lactic acid bacteria*" perhaps implies that these bacteria have a somewhat simple metabolism, resulting in one or a few fermentation end products. This may be the case in the laboratory environment that we often impose on them. However, it is clear that LAB have a very diverse metabolic ability to adapt to a variety of conditions. The physiology section of this chapter will describe the main features of LAB, such as carbohydrate metabolism and bioenergetics. Some emphasis will be placed on the variations of the general "theme" of metabolism that may occur under certain conditions.

This volume concerns the technological, nutritional, and health aspects of LAB. This reflects the intimate association of the term with food and feed manufacture. Again, this is perhaps more of a historical tradition than a scientifically reached position, since the group includes bacteria that are highly pathogenic and therefore undesirable in food (e.g., many streptococci). In addition, some lactobacilli and other LAB generally associated with food have been implicated in disease;[27] carnobacteria are normal inhabitants in meat, but are also fish pathogens.[28] There are more examples of the "dual" nature of LAB as a group. The main emphasis in this chapter will be on LAB *normally* associated with food manufacture and positive health aspects.

III. CLASSIFICATION OF LACTIC ACID BACTERIA

A. General Description and Included Genera

An unequivocal definition of the term lactic acid bacteria is not possible to give. Inevitably, most characteristics used in such a definition are subject to more or less qualification,[4] meaning that they are accurate only under "normal" or "standard" conditions and that exceptions to the definition can be found. Therefore, it is more appropriate to describe the *typical* lactic acid bacterium, which is gram-positive, non–spore-forming, catalase-negative, devoid of cytochromes, of nonaerobic habit but aerotolerant, fastidious, acid-tolerant, and strictly fermentative, with lactic acid as the major end product during sugar fermentation. LAB are generally associated with habitats rich in nutrients, such as various food products (milk, meat, beverages, vegetables), but some are also members of the normal flora of the mouth, intestine, and vagina of mammals. Variations of this general theme are common. It is really only the gram-positive characteristic that cannot be challenged. For instance, catalase and cytochromes may be formed by some LAB on certain media (see below), some streptococci (e.g., *Streptococcus bovis*) have quite limited nutritional requirements, sugar fermentation may result in very little lactic acid under certain conditions, etc. Furthermore, there are no strong scientific arguments for excluding spore-forming bacteria, which otherwise resemble LAB (i.e., *Sporolactobacillus*), since some of the genera we consider "genuine" LAB are not clearly separated from these phylogenetically. In this chapter I will follow the historical tradition not to include spore-formers in the LAB group. The above definition, despite its limitations, is useful as a core or center around which the actual descriptions of genera or species are formulated. A key feature of LAB that must be emphasized is the inability to synthesize porphyrin groups (e.g., heme). This is the actual physiological background for some of the characteristics mentioned above. This makes LAB devoid of a "true" catalase and cytochromes when grown in laboratory growth media, which lack heme or related compounds. Under these conditions, considered normal in most studies of these bacteria, LAB do not possess the mechanism of an electron transport chain and rely on fermentation, i.e., substrate-level phosphorylation, for generating energy. Since catalase activity, mediated by a nonheme "pseudocatalase," can occur in some LAB,[29] the lack of cytochromes may be a more reliable characteristic for preliminary diagnosis than the commonly used catalase test.[4] However, it is important to note that the situation may be totally different if heme (or hemoglobin) is added to the growth medium. A true catalase and even cytochromes may be formed, in some cases resulting in respiration with a functional electron transport chain.[30–37]

It is appropriate to start the introduction to the LAB genera with *Bergey's Manual* of 1986, since this edition essentially is the last in a continuoum reflecting the historical tradition dating back to the work of Orla-Jensen (1919). The genera that then fit the general description of the typical LAB were *Aerococcus* (*A.*), *Lactobacillus* (*Lb.*), *Leuconostoc* (*Ln.*), *Pediococcus* (*P.*), and *Streptococcus* (*S.*). The genus *Bifidobacterium* is historically also considered to belong to the LAB group. In the 7th edition of *Bergey's Manual* of 1957, the bifidobacteria were designated *Lb. bifidum.* Although *Bifidobacterium* species do fit the general description above, they are phylogenetically more related to the Actinomycetaceae group of gram-positive bacteria. In addition, they have a special pathway for sugar fermentation, unique to the genus, which clearly separates them from the LAB group. Bifidobacteria will, therefore, not be considered in this general overview of LAB. However, due to their significance in the gastrointestinal tract of animals and humans and possible probiotic action, bifidobacteria are described in more detail elsewhere in this book. Major revisions of the taxonomy of LAB, in particular of the streptococci, were anticipated in *Bergey's Manual* of 1986[38,39] and to some extent already realized by the year of that issue. Thus, the former genus *Streptococcus* was first divided into three: *Enterococcus* (*E.*), *Lactococcus* (*Lc.*), and *Streptococcus sensu stricto*.[40–42] Later, some motile LAB, otherwise resembling lactococci, were suggested to form a separate genus, *Vagococcus* (*V.*).[43] The genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* have largely remained unchanged, but some rod-shaped LAB, previously included in *Lactobacillus*, now form the genus *Carnobacterium* (*C.*),[28] and the former species *Pediococcus halophilus* has been raised to genus level, forming the genus *Tetragenococcus* (*T.*).[11] A distinct phylogenetic cluster of heterofermentative LAB, including species previously assigned to either *Lactobacillus* or *Leuconostoc*, has been suggested to form a separate genus, *Weissella* (*W.*).[13] *Leuconostoc oenos*, the "wine leuconostocs," has been proposed to form a genus of its own, *Oenococcus* (*O.*).[44] New genera, e.g., *Alloiococcus*, *Dolosicoccus*, *Dolosigranulum*, *Eremococcus*, *Facklamia*, *Globicatella*, *Helcococcus*, *Ignavigranum*, and *Lactosphaera*, have also been described to include some strains that were shown to be related to the LAB group, both physiologically and phylogenetically.[45–52] These represent special cases and will not be dealt with further. The revisions made since 1986 are supported by extensive chemotaxonomic and genetic data.

B. Classification at the Genus Level

As mentioned, the general basis for the classification of LAB in different genera has remained largely unchanged since the work of Orla-Jensen.[3] However, with the description of new genera and species it is becoming increasingly difficult to use these classical tests for reliable genus identification. Still, these phenotypic characteristics are useful as a starting point for more sophisticated tests. Although morphology is regarded as questionable as a key characteristic in bacterial taxonomy,[10] it is still important in the current descriptions of the LAB genera. Thus, LAB can be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). One exception is the relatively recently described genus *Weissella*, which is the first genus in the LAB group that, by definition, can include both cocci and rods.[13] Furthermore, cell division in two perpendicular directions in a single plane [previously incorrectly described as "division in two planes"[53]], leading to tetrad formation, is used as a key characteristic in the differentiation of the cocci. The tetrad-forming genera are *Aerococcus*, *Pediococcus*, and *Tetragenococcus.*

An important characteristic used in the differentiation of the LAB genera is the mode of glucose fermentation under standard conditions, i.e., nonlimiting concentrations of glucose and growth factors (amino acids, vitamins, and nucleic acid precursors) and limited oxygen availability. Under these conditions LAB can be divided into two groups: the homofermentative, converting glucose almost quantitatively to lactic acid and the heterofermentative, fermenting glucose to lactic acid, ethanol/acetic acid, and CO_2. In practice, a test for gas production from glucose will distinguish between the groups.[54] (For a more detailed discussion concerning the metabolic pathways, see Sec. IV.A.) Leuconostocs, oenococci, weissellas, and a subgroup of lactobacilli are heterofermentative; all other LAB are homofermentative.

Growth at certain temperatures is used mainly to distinguish between some of the cocci. The "classical" enterococci grow at both 10°C and 45°C, lactococci and vagococci at 10°C, but not at 45°C. Streptococci generally do not grow at 10°C, while growth at 45°C is dependent on the species. Salt tolerance (6.5% NaCl) may also be used to distinguish between enterococci, lactococci/vagococci, and streptococci, although variable reactions can be found among streptococci.[55] Extreme salt tolerance (18% NaCl) is confined to the genus *Tetragenococcus.* Tolerance to acid and/or alkaline conditions may also be useful. Aerococci, carnobacteria, enterococci, tetragenococci, and vagococci are characterized by growth at relatively high pH, although not all can grow at the standard test pH of 9.6. Formation of the different isomeric forms of lactic acid during fermentation of glucose can be used to distinguish between leuconostocs and most heterofermentative lactobacilli, as the former produce only D-lactic acid and the latter a racemate (DL-lactic acid), but *Weissella* strains may cause confusion in this regard.

A summary of the differentiation of LAB genera with classical phenotypic tests is shown in Table 1. The genus *Carnobacterium* is indistinguishable from *Lactobacillus* with these tests, as is *Vagococcus* from *Lactococcus. Vagococcus* and *Carnobacterium* have unique fatty acid compositions, which separate these genera from most other LAB.[28,43] In general, carnobacteria can be distinguished from lactobacilli by their ability to grow at pH 9.0 and inability to grow on acetate media selective for lactobacilli. Pediococci can be confused with aerococci, since the morphology is similar. However, pediococci are more acid-tolerant than aerococci and grow well anaerobically, contrary to the more microaerophilic nature of aerococci.[56] *Weissella* species can easily be confused with leuconstocs or heterofermentative lactobacilli. Oenococci fall into the *Leuconostoc* group with the classical tests but are easily distinguished by their extreme acid and ethanol tolerance.[44] It should be noted that there are phenotypic overlaps between genera and exceptions to the general rules outlined in Table 1 can be found. For example, the genus *Enterococcus* contains many species that do not conform to the classical tests.[57,58] Classification of LAB is becoming dependent on more sophisticated methods, of which direct rRNA sequencing is the most accurate at the genus level. Known rRNA sequences have also been used to develop genus-specific probes.[13,59]

C. Classification at Species Level

It is impossible within the scope of this review to describe the classification of all species of LAB. The genus *Lactobacillus* alone includes about 80 recognized species.[60] Therefore, the following section will only be a summary, concentrating on the means by which classification within a genus can be done and mentioning some of the most interesting species from a food technology point of view. For the most recent, comprehensive review on

Table 1 Differential Characteristics of Lactic Acid Bacteria

	Rods		Cocci							
Character	*Carnob.*	*Lactob.*	*Aeroc.*	*Enteroc.*	*Lactoc. Vagoc.*	*Leucon. Oenoc.*	*Pedioc.*	*Streptoc.*	*Tetragenoc.*	*Weissella* [a]
Tetrad formation	−	−	+	−	−	−	+	−	+	−
CO_2 from glucose[b]	−[c]	±	−	−	−	+	−	−	−	+
Growth at 10°C	+	±	+	+	+	+	±	−	+	+
Growth at 45°C	−	±	−	+	−	−	±	±	−	−
Growth in 6.5% NaCl	ND[d]	±	+	+	−	±	±	−	+	±
Growth in 18% NaCl	−	−	−	−	−	−	−	−	+	−
Growth at pH 4.4	ND	±	−	+	±	±	+	−	−	±
Growth at pH 9.6	−	−	+	+	−	−	−	−	+	−
Lactic acid[e]	L	D, L, DL[f]	L	L	L	D	L, DL[f]	L	L	D, DL[f]

+, positive; −, negative; ±, response varies between species; ND, not determined.

[a]*Weissella* strains may also be rod-shaped.

[b]Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.

[c]Small amounts of CO_2 can be produced, depending on media.

[d]No growth in 8% NaCl has been reported.

[e]Configuration of lactic acid produced from glucose.

[f]Production of D-, L-, or DL-lactic acid varies between species.

the taxonomy of LAB, the reader is referred to Volume 2 in the Lactic Acid Bacteria series edited by B. J. B. Wood,[5] which also includes descriptions of the individual species. A review by Stiles and Holzapfel[61] is also very useful. At the time of this writing, the online publication "The Prokaryotes"[62] contains recently updated chapters for lactococci, enterococci, some streptococci, and the "leuconostoc-like" LAB[58,63–66] and will certainly be an even more valuable resource in the future as more chapters are updated.

As indicated previously, proper classification of LAB is beginning to rely on molecular biology methods, although some of Orla-Jensen's concepts are still viable. This is perhaps more true regarding classification at the species than at the genus level. In some cases, only an analysis at the nucleic acid level will resolve classification problems. Still, the classical phenotypic/biochemical characterization is important for a preliminary classification as well as learning about the properties of the strains. Some of the characteristics listed in Table 1 are useful also in the classification at species level, e.g., salt and pH tolerance, growth at certain temperatures, and configuration of the lactic acid produced. Other characteristics used in the phenotypic/biochemical characterization of strains are range of carbohydrates fermented, arginine hydrolysis, acetoin formation (Voges-Proskauer test), bile tolerance, type of hemolysis, production of extracellular polysaccharides, growth factor requirements, presence of certain enzymes (e.g., β-galactosidase and β-glucuronidase), growth characteristics in milk, and serological typing. Further characterization includes more molecular/chemotaxonomic approaches, including type of diamino acid in the peptidoglycan, presence and type of teichoic acid, presence and type of menaquinones, guanine + cytosine (G + C) ratio of the DNA, fatty acid composition, and electrophoretic mobility of the lactate dehydrogenase (LDH).

1. *Enterococcus*, *Lactococcus*, *Streptococcus*, and *Vagococcus*

As mentioned, the genera *Enterococcus*, *Lactococcus*, *Streptococcus*, and *Vagococcus* were earlier included in one genus, *Streptococcus.* For details regarding the major taxonomic revision of the "streptococci," in the mid-1980s, the reader is referred to a review by Schleifer and Kilpper-Bälz,[67] which summarizes the phenotypical, biochemical, and molecular characteristics of the genera.

Historically, serological typing with the Lancefield grouping[68] has been very important in the classification of streptococci. The method is now considered to be less important in classification, but still very useful in the rapid identification of major pathogens.[38,42,54,64] However, there is undoubtedly some correlation between the presence of the group D antigen and the "classical" enterococci. Similarily, the group N antigen is correlated with lactococci, but note that the vagococci also possess the group N antigen.[43]

Despite the formation of new genera, the genus *Streptococcus sensu stricto* is still very large and the classification is difficult. The genus is broadly divided into three groups: pyogenic, oral, and "other" streptococci.[69] Some anaerobic cocci, previously included in the genus as the group "anaerobic streptococci,"[38] were shown to be unrelated to all other streptococci and have been excluded.[42] The pyogenic group contains several famous pathogens, e.g., *S. pyogenes* and *S. agalactiae.* Another pathogen, *S. pneumoniae*, was earlier included in this group, but has been transferred to the oral group, which contains species mostly associated with the oral cavity of humans and animals. Some oral streptococci, e.g., *S. mutans*, can be causative agents of dental caries, others of infective endocarditis.[38] As a general rule, the pyogenic streptococci are β-hemolytic, while the oral streptococci are α- or nonhemolytic, but exceptions can be found. The sequence of 16S

rRNA has been determined for most species,[70,71] and this has clarified the intrageneric phylogeny. These studies suggest that the oral group can be divided in five phylogenetic subgroups: the *anginosus*, the *mitis*, the *salivarius*, the *bovis*, and the *mutans* groups, respectively. Biochemical characteristics, such as carbohydrate fermentation, hydrolysis of arginine, and certain enzyme activities, are used in classification schemes. Genetic fingerprinting techniques, such as RFLP, REP-PCR, and RAPD (see Sec. III.E) have also been employed in the identification and classification of streptococci.[65]

Essentially the only streptococcal species associated with food technology is *S. thermophilus*, which is used in the manufacture of yogurt (in coculture with *Lb. delbrückii* subsp. *bulgaricus*) and certain cheeses. *S. thermophilus* was included in the group "other streptococci" by Schleifer and Kilpper-Bälz[67] and Hardie,[72] but is now in the oral group.[69] A close relationship at the DNA level with *S. salivarius* has been established.[72,73] Farrow and Collins[73] proposed that *S. thermophilus* be considered a subspecies of *S. salivarius*, since DNA-DNA homology values of greater than 70% were determined. Thus, for some time the name *S. salivarius* subsp. *thermophilus* was valid. However, the suggestion was later rejected,[42] since an investigation of a large number of strains revealed lower DNA-DNA homology values for some strains. In addition, the large phenotypic differences would justify two separate species. A third species, *S. vestibularis*, also belongs to this closely related group of streptococci.[71] Heat resistance, the ability to grow at 52°C, and the rather limited number of carbohydrates attacked distinguish *S. thermophilus* from most other streptococci.[72] A species-specific probe for *S. thermophilus* has been developed.[74]

Lactococci are intimately associated with dairy products, but out of the five species currently recognized,[66] only one, *Lc. lactis*, is actually used in dairy technology. *Lc. garviae* strains have been associated with bovine mastitis and fish lactococcosis.[66] Three subspecies of *Lc. lactis* can be distinguished: *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, and *Lc. lactis* subsp. *hordniae.* Only the first two are important in dairy manufacture. *Lc. lactis* subsp. *lactis* includes species formerly designated *S. lactis* subsp. *lactis*, *S. lactis* subsp. *diacetylactis*, and *Lactobacillus xylosus*.[41] The latter illustrates the fact that morphology can be deceptive in classification of LAB, i.e., the distinction between cocci and rods is not always an easy task. *Lc. lactis* subsp. *cremoris* includes species previously designated *S. cremoris* or *S. lactis* subsp. *cremoris*. *Lc. lactis* subsp. *cremoris* is distinguished from *Lc. lactis* subsp. *lactis* by the inability to (a) grow at 40°C, (b) grow in 4% NaCl, (c) hydrolyze arginine, and (d) ferment ribose.[41] The *lactis* and *cremoris* subspecies of *Lc. lactis* have also been shown to be genetically distinct by DNA-DNA homology studies[75] and comparison of 16S rRNA sequences.[76] However, it should be noted that some strains of the subspecies *lactis* phenotype are genetically subspecies *cremoris*, among them the laboratory strains commonly used worldwide, MG1363 and LM0230.[77] Common biochemical characteristics (e.g., sugar utilization) can be used to distinguish between the species of lactococci, but genetic methods are also available[76–80] (see Sec. III.E).

As noted, species of the newly described genus *Vagococcus* are easily confused with lactococci, but the genera are clearly distinguished by fatty acid composition. Some, but not all strains of vagococci are motile.[43,81] Genus- and species-specific oligonucleotide probes are available for vagococci,[82] which make a reliable identification of these bacteria feasible.

Enterococci are not considered to be of particular importance in food technology. Some species, in particular *E. faecalis* (previously *S. faecalis*), can be opportunistic

pathogens[83,84] and are, therefore, generally undesirable in food. There is also some concern about the propensity of fecal enterococci to be resistent to antibiotics and to transfer such traits by means of mobile genetic elements.[85,86] Vancomycin resistance and the conjugative transfer of the trait have received special attention in this regard due to the "last resort" status of vancomycin in the treatment of patients infected with multiresistant staphylococci.[87] However, preparations of *E. faecium* (previously *S. faecium*) and *E. faecalis* have been used as probiotics[88,89] and as silage inoculants.[90] The probiotic approach is not far-fetched, since the natural habitat of many enterococci is the intestines of humans and animals.[55] Species of enterococci have also been shown to be present in some local types of cheeses in southern Europe.[91] As mentioned, it is difficult, if not impossible, to definitely assign a strain to the genus with the tests shown in Table 1. Useful additional tests for which a majority of enterococci are positive are the Voges-Proskauer test and fermentation of ribose.[58] With only phenotypic tests, one probably has to identify strains to species level to unambigously show that they are enterococci. Species are differentiated mainly by carbohydrate fermentation patterns, arginine and hippurate hydrolysis, and presence and/or type of menaquinones.[67] A number of genetic methods are also available.[58,92] For further information on identification of enterococci see Devriese et al.[57,58] *E. faecalis* and *E. hirae* (in older publications: *S. feacalis* or *S. faecium*) have been of great value for general LAB research in being model organisms in physiological studies of, for instance, bioenergetics and membrane biology.[21,23,24]

2. *Aerococcus*, *Pediococcus*, and *Tetragenococcus*

Aerococci, pediococci, and tetragenococci constitute the tetradforming LAB. The genus *Aerococcus* currently contains five species. Aerococci are generally of minor interest in food technology. However, a recent report suggests that some aerococci could be responsible for greening of cooked meat products.[93] Information on the genus *Aerococcus* is given in a review by Weiss.[94]

With the transfer of *P. urinae-equi* to *Aerococcus* and *P. halophilus* to *Tetragenococcus*, the genus *Pediococcus* can be described as "the only acidophilic, homofermentative, lactic acid bacteria that divide alternately in two perpendicular directions to form tetrads."[95] Pediococci are important in food technology in both a negative and positive sense. *P. damnosus* is a major spoilage organism in beer manufacture, since growth may lead to diacetyl/acetoin formation, resulting in a buttery taste.[96] *P. acidilactici* and *P. pentosaceus* are used as starter cultures for sausage making and as silage inoculants.[90,97] Pediococci may also be important constituents of the complex known as the nonstarter lactic acid bacteria (NSLAB), which is involved in the ripening of cheese.[98,99] The main characteristics for distinguishing between the species are the range of sugars fermented, hydrolysis of arginine, growth at different pH levels (7.0 and 4.5), and the configuration of lactic acid produced.[95,96] *P. pentosaceus* and *P. acidilactici* are difficult to distinguish using these characteristics but have been shown to be distinct species with DNA-DNA homology studies.[96] These species are also similar in that they may produce a nonheme "pseudocatalase."[29] Genetic fingerprinting methods are also available for distinguishing pediococci.[100]

As mentioned, the genus *Tetragenococcus* contains strains previously regarded as *P. halophilus.* Only two species, *T. halophilus* and *T. muriaticus*, are currently recognized, but one enterococcal species, *E. solitarius*, has been shown to be related to *Tetragenococcus* phylogenetically.[101] In addition to extreme salt tolerance (>18% NaCl), which distinguishes them from other LAB, tetragenococci generally require salt

in the range of 5% NaCl for growth.[96] *Tetragenococcus* species are important in lactic fermentation of high-salt-containing food, e.g., soy sauce.[96,102,103]

3. *Leuconostoc*, *Oenococcus*, and *Weissella*

The genus *Leuconostoc* was previously defined as being heterofermentative, coccoid LAB producing only D-lactic acid from glucose and not producing ammonia from arginine. The leuconostocs were thus separated from other cocci of the LAB by their heterofermentative metabolism and from heterofermentative lactobacilli by morphology and some key traits. It was, however, easy to confuse leuconostocs with some "coccoid rods" of the heterofermentative lactobacilli. Phylogenetic analysis of the leuconostocs revealed considerable heterogeneity of the genus.[104,105] It was anticipated that *Ln. paramesenteroides* together with some heterofermentative lactobacilli (e.g., *Lb. confusus* and *Lb. viridescens*) could represent the nucleus of a new genus, since this group was separated from both other leuconostocs and lactobacilli. Later, other heterofermentative LAB falling into this group were isolated from meat sources, and the genus *Weissella* was suggested to comprise these "leuconostoc-like" bacteria.[13] As mentioned, *Weissella* therefore includes both cocci and rods. These phylogenetic studies also revealed that the so-called wine leuconostocs, alloted to the species *Ln. oenos*, were only distantly related to other leuconostocs and that this species therefore warranted a separate genus.[104,105] Later, the genus *Oenococcus* was proposed for these bacteria.[44] Thus, *Ln. oenos* is now designated *O. oeni.* It is important to note that although these taxonomic revisions were necessary from a phylogenetic point of view, they do not make the practical classification of "leuconostoc-like" LAB simpler than before. Oenococci are easy distinguishable by their extreme acid and ethanol tolerance, but separating *Weissella* from *Leuconostoc sensu stricto* and from some heterofermentative lactobacilli is still problematic. For practical reasons it is best to treat the "leuconostoc-like" LAB as a group and use confirmatory tests to definitely assign genus and species status for a particular strain. The most reliable method to determine if a strain belongs to *Weissella* is probably to use a genus-specific rRNA probe.[13] For rapid identification of *Weissella* species, an amplified ribosomal DNA restriction analysis (ARDRA) was recently developed. This method employs both PCR with genus-specific primers and restriction pattern analysis for species determination.[106]

Leuconostocs may form significant amounts of diacetyl from citrate in milk, and some species, mainly *Ln. mesenteroides* subsp. *cremoris*, have been used in the dairy industry for this purpose. Leuconostocs are also important in spontaneous vegetable fermentations, e.g., sauerkraut, where they often initiate the lactic fermentation.[107] Many *Weissella* species seem to be associated with meat, where they may proliferate at low temperatures.[13] Recently, and somewhat surprisingly, a bacterium isolated from garden soil was shown to represent a new species of *Weissella*.[108]

Species and subspecies of *Leuconostoc* and *Weissella* are distinguished by characteristics such as carbohydrate fermentation patterns, dextran formation (from sucrose), hydrolysis of esculine, growth requirements, growth at different pH and temperatures, and dissimilation of citrate and/or malate, but classification is difficult.[13,63,109,110] The dissimilation of malate requires attention, as this metabolism results in L-lactic acid.[111] Hence, care must be taken that malate is not present in the growth medium when the configuration of the lactic acid (from glucose fermentation) is to be determined.[109] Ribotyping and PCR-based methods (see Sec. III.E) have also been used successfully for distinguishing the *Leuconostoc*-like LAB.[63]

4. *Lactobacillus* and *Carnobacterium*

The genus *Lactobacillus* is by far the largest of the genera included in LAB. It is also very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. The heterogeneity is reflected by the range of mol% G + C of the DNA of species included in the genus. This range is 32–55%, twice the span usually accepted for a single genus.[112] The heterogeneity and the large number of species are due to the definition of the genus, which essentially is rod-shaped lactic acid bacteria. Such a definition is comparable to an arrangement where all the coccoid LAB were included in one genus. However, among the cocci, phenotypic traits were early recognized, which made differentiation into several genera possible. Even if the situation was more difficult for the rod-shaped LAB, Orla-Jensen (1919) essentially tried to divide this group in a way similar to that with the cocci. Thus, the subgenera of *Lactobacillus* were created: *Thermobacterium*, *Streptobacterium*, and *Betabacterium.* Remarkably, this division is still valid to a considerable degree, although the designations have been dropped and some modifications in the definitions of the subgroups have been made.[113,114] Table 2 shows a summary of the characteristics used to distinguish between the three groups and some of the more well-known species included in each group. The physiological basis for the division is (generally) the presence or absence of the key enzymes of homo- and heterofermentative sugar metabolism, fructose-1,6-diphosphate aldolase and phosphoketolase, respectively.[113,115,116] (For further details regarding the division of LAB in homo- and heterofermentative, see Sec. IV.A.) In the most recent comprehensive description of the genus,[114] the physiological division into the three groups (A, B, and C) was kept, but each species was also assigned a suffix (a, b, or c) to reflect the position in certain phylogenetic clusters (see below). For example, *Lb. acidophilus* was placed in group Aa and *Lb. salivarius* in group Ab, since these species belong to different phylogenetic clusters. Note that the cluster c contains those heterofermentative lactobacilli that now should be designated *Weissella*, e.g., *Lb. confusus* and *Lb. viridescens* (see above).

Table 2 Arrangement of the Genus *Lactobacillus*

Characteristic	Group I, Obligately homofermentative	Group II, Facultatively heterofermentative	Group III, Obligately heterofermentative
Pentose fermentation	−	+	−
CO_2 from glucose	−	−	+
CO_2 from gluconate	−	+[a]	+[a]
FDP aldolase present	+	+	−
Phosphoketolase present	−	+[b]	+
	Lb. acidophilus	*Lb. casei*	*Lb. brevis*
	Lb. delbrückii	*Lb. curvatus*	*Lb. buchneri*
	Lb. helveticus	*Lb. plantarum*	*Lb. fermentum*
	Lb. salivarius	*Lb. sakei*	*Lb. reuteri*

[a]When fermented.
[b]Inducible by pentoses.
Source: Adapted from Sharpe [117] and Kandler and Weiss [113].

The classical ways of distinguishing between species of lactobacilli have been carbohydrate fermentation patterns, configuration of lactic acid produced, hydrolysis of arginine, growth requirements, and growth at certain temperatures.[54,117] These characteristics are still useful, but proper classification may also require analysis of the peptodoglycan, electrophoretic mobility of the LDH, mol% G + C of the DNA, and DNA-DNA homology studies.[113,116] Species-specific oligonucleotide probes (derived from rRNA sequences) for a growing number of lactobacilli are now available,[81] possibly providing simpler classification schemes. Different PCR techniques and direct sequencing of PCR fragments (e.g., of rRNA genes) are also offering new possibilities for rapid identification and classification of lactobacilli and LAB in general (see Sec. III.E).

Lactobacilli are widespread in nature, and many species have found applications in the food industry. They are generally the most acid-tolerant of the LAB[21] and will, therefore, terminate many spontaneous lactic fermentations such as silage and vegetable fermentations.[107] Lactobacilli are also associated with the oral cavity, gastrointestinal tract, and vagina of humans and animals.[113,117] Some species, e.g., *Lb. brevis*, *Lb. casei*, and *Lb. plantarum*, can be found in many habitats. Others are more specialized and are found only in certain niches, e.g., the sourdough organism *Lb. sanfransiscensis* and the yogurt-associated *Lb. delbrueckii* subsp. *bulgaricus* (previously *Lb. bulgaricus*).

Due to the scope of this chapter, details of the classification of species of lactobacilli are not discussed further. For detailed information on this topic, the reader is referred to the previously mentioned review by Hammes and Vogel.[114] Note, however, that new species are constantly being described. In the first few years of the new millenium (2000–2002), approximately 20 new *Lactobacillus* species were validly published. As mentioned, it has been known for some time that the genus *Lactobacillus* and its division into the three groups shown in Table 2 are not in accord with natural relationships revealed by phylogenetic analysis[113,118,119] (see below). However, it is not easy to envisage how a new classification system would appear, since it would be difficult to find properties other than rRNA sequences that would constitute a basis for a phylogenetically correct taxonomy.

Species of the genus *Carnobacterium* were originally classified as group III lactobacilli under the designations *Lb. divergens, Lb. carnis*, and *Lb. piscicola*.[28,113] Later studies showed that these bacteria were separate from lactobacilli and warranted a separate genus[28] and that the metabolism of glucose was predominantly homofermentative.[120] Generally, carnobacteria grow at relatively high pH (e.g., pH 9), while lactobacilli do not.[121] Furthermore, the fatty acid composition of carnobacteria differs from that of lactobacilli.[28] Carnobacteria are characteristically found in meat and meat products, where they are able to proliferate even at low temperatures.[28] A simple identification key, confirmed by DNA-DNA hybridization, for distinguishing between *C. divergens, C. piscicola*, and typical meat-associated lactobacilli has been published.[122] In addition, a genus-specific probe has been developed.[59] A rapid method for identification of *Carnobacterium* isolates from food, based on restriction fragment length polymorphism (RFLP) of PCR-amplified 16S–23S ribosomal intergenic spacer regions (ISRs), was recently developed.[123]

D. Phylogeny of the Lactic Acid Bacteria

Comparisons of the sequence of rRNAs is now regarded to be the optimal measure for determining true phylogenetic relations among bacteria.[10] Initially, these comparisons were made by DNA-rRNA hybridizations or oligonucleotide cataloguing (i.e., sequencing

of cleavage products of rRNA). Advances in molecular genetics techniques have led to methods for sequencing long stretches of rRNA, first by the use of reverse transcriptase,[8] but now by direct PCR sequencing of the rRNA genes. The computerized methods now available for handling large amounts of sequence data have made it possible to construct meaningful phylogenetic trees of the entire bacterial kingdom as well as details of certain parts of it.[10]

From the data obtained from both oligonucleotide cataloguing and rRNA sequencing, it has been shown that the gram-positive type cell wall has a relatively strong phylogenetic relevance. The gram-positive bacteria cluster in 2 of the 12 major eubacterial phyla (but not all bacteria in these phyla have a gram-positive cell-wall). It is common to designate the 2 gram-positive phyla the high-G + C and the low-G + C subdivision, which reflects the mol% G + C in the DNA. The "splitpoint" is often set at 50%, but is rather an interval around 53–55%, since some species (e.g., *Lb. fermentum* and *Lb. pontis*) clearly belonging to the low-G + C subdivision have a G + C content in that range. The high-G + C or Actinomycetes subdivision encompasses genera such as *Bifidobacterium*, *Arthrobacter*, *Micrococcus*, *Propionibacterium*, *Microbacterium*, *Corynebacterium*, *Actinomyces*, and *Streptomyces*.[10,118] The low-G + C or the *Clostridium* subdivison includes all LAB, together with aerobes and facultative anaerobes like *Bacillus*, *Staphylococus*, and *Listeria* and anaerobes such as *Clostridium*, *Peptococcus*, and *Ruminococcus*.[10,118]

The details of the phylogenetic relationships between the LAB genera and also between LAB and other genera of the low-G + C subdivision were revealed mainly by the extensive work of M.D. Collins with coworkers (but also others) using both reverse transcriptase and PCR sequencing techniques.[11–13,43,70,71,101,105,119,124,125] The LAB have been considered to form a "supercluster," which lies phylogenetically between the strictly anaerobic species (e.g., clostridia) and facultatively or strictly aerobic species (e.g., staphylococci and bacilli), in accord with their lifestyle, i.e., "on the threshhold of anaerobic and aerobic life".[113,116] This is not entirely true. The schematic phylogenetic tree of the LAB as a group, shown in Fig. 1, and the following discussion is based on the work by Collins and coworkers.

As indicated, most genera of the LAB are now partly defined and based on phylogenetic measurements. This is the case for *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Leuconostoc sensu stricto*, *Oenococcus*, *Streptococcus sensu stricto*, *Tetragenococcus*, *Vagococcus*, and *Weissella*, each of which forms a coherent phylogenetic unit. Among these, a certain clustering is evident. Thus, *Carnobacterium*, *Enterococcus*, and *Vagococcus* form a tight cluster and are more related to each other than to any other LAB. The genera *Aerococcus* and *Tetragenococcus* could be included in this cluster as peripheral members. Furthermore, this group of genera appears somewhat closer phylogenetically to aerobes and facultative anaerobes of the low-G + C subdivision than to the remaining LAB. The genera *Lactococcus* and *Streptococcus* are also more related to each other than to other LAB, although this tendency is not as strong as for the *Enterococcus* group. The "leuconostoc-like" LAB, i.e., *Weisella* and *Leuconostoc sensu stricto*, are clearly related, and *Oenococcus* also belong to this branch, although the evolutionary distance to this genus is large. It has been discussed whether *Oenococcus* represents a case of a rapidly evolving organism.[44,104] Since the closest relatives of *Oenococcus* seem to be the "leuconostoc-like" LAB,[105,124] both phylogenetically and physiologically, one can imagine that the adaptation to an unusual habitat (wine) has required extensive changes in the genome, resulting in a rapid divergence from related species.

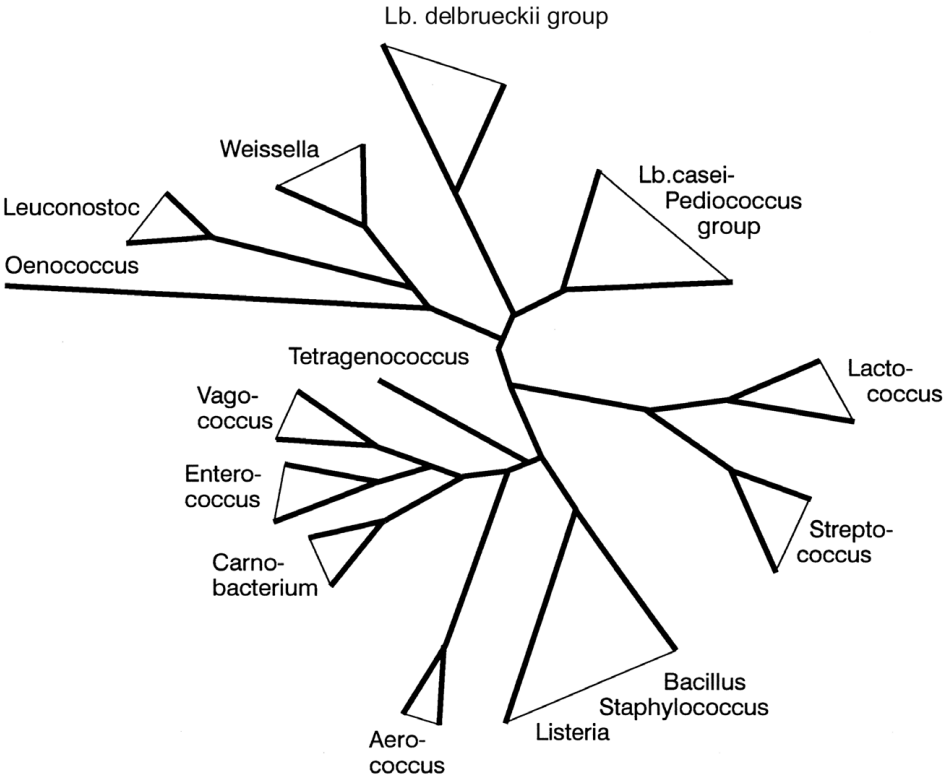

Figure 1 Schematic, unrooted phylogenetic tree of the lactic acid bacteria, including some aerobic and facultatively anaerobic gram-positives of the low G + C subdivision. *Note*: Evolutionary distances are approximate.

The remaining LAB genera, *Lactobacillus* and *Pediococcus*, form a supercluster within LAB, which can be divided into two subclusters, possibly each worthy of genus status.[125] These clusters do not correlate with the physiological subdivision of the genus *Lactobacillus* as described above (see Table 2). The clusters are (a) the *Lb. delbrueckii* group, which contains many, but notably not all, of the obligately homofermentative (group I) lactobacilli (e.g., *Lb. delbrueckii* with subspecies, *Lb. acidophilus*, *Lb. helveticus*, and *Lb. jensenii*) and a few facultatively heterofermentative (group II) lactobacilli and (b) the *Lb. casei–Pediococcus* group, comprising all other lacobacilli, i.e., the remaining obligately homofermentative, all heterofermentative, and most of the facultatively heterofermentative lactobacilli [note that some species, previously regarded as heterofermentative lactobacilli, now belong to the genus *Weissella* [13]]. In addition, as the designation indicates, included in the latter group are all species of *Pediococcus.* All pediococci except *P. dextrinicus* form a tight cluster within the *Lb. casei–Pediococcus* group.

What conclusions can be drawn from the phylogenetic investigations of LAB? One obvious conclusion, which is no surprise, is that morphology is a poor indicator of relatedness, as shown by the phylogenetic structure of *Lactobacillus-Pediococcus* and perfectly illustrated by the new genus *Weissella.* Another conclusion is that future taxonomic

revisions are still needed, although much has been done during the last 15 years to clarify natural relations and to define genera from such analyses. The main remaining problem is how to deal with the genera *Lactobacilus* and *Pediococcus.* As pointed out by Woese [10], it is preferable that there be some correlation between the phylogenetic position of a certain strain or species and its phenotype. The problem lies in how to find the right characteristic(s) that will correlate with true relationships. In 1983 it was suggested that pediococci be included in an "extended" *Lactobacillus* genus.[126] Phylogenetically this could be correct, but would it be generally accepted? Another solution would be to define smaller phylogenetic clusters as new genera. Consequently, many typical lactobacilli such as *Lb. acidophilus*, *Lb. plantarum*, and *Lb. fermentum* would end up in different genera.

As a group, LAB are not clearly separated from all other groups of the low-G + C subdivision of the gram-positives. In particular, it seems that some LAB "overlap" with some of the more aerobic genera such as *Bacillus*, *Listeria*, and *Staphylococcus.* This has recently been supported by genome sequencing.[19,20] No comprehensive rRNA sequence comparisons between LAB and the strict anaerobes have been made. However, scattered reports including some rRNA sequences and earlier oligonucleotide cataloguing[118] suggest that the evolutionary distance between these groups is quite large. However, it is not clear whether the *Enterococcus* group is less related to the strict anaerobes than, for instance, the *Lb. casei–Pediococcus* group. Therefore, the statement that LAB are phylogenetically positioned between the strict anaerobes and the aerobes in accordance with their lifestyle is not correct. Rather, LAB seem to be phylogenetically intermixed with the aerobic and facultatively anaerobic genera of the low-G + C subdivision of gram-positive bacteria.

E. New Tools for Classification and Identification

The classification of LAB, described above, is largely based on phenotypic and biochemical characters. In practice, in the routine identification of isolates, these characteristics may not be enough to definitely assign a strain to a particular species. Today, with the availability of rapid and automatic DNA sequencing technology, direct sequencing of the 16S rRNA gene has emerged as the most powerful and relatively easy one-step method for classification of bacteria.

The determination of 16S rRNA sequences for the elucidation of the phylogeny of the LAB around 1990 (see above) initiated a rapid development of DNA probes for identification of these bacteria. Before that there had been some attempts to develop DNA probes based on selected fragments of a DNA library, e.g., for *Lb. curvatus*,[127] *Lb. delbrueckii*,[128] and *Lb. helveticus*.[129] Identification of LAB with the use of 16S (or 23S rRNA)-targeted probes was developed and used for lactococci and enterococci,[78,130] lactobacilli from different niches,[131–133] carnobacteria from meat,[134] distinguishing vagococci from other LAB,[82] *S. thermophilus*,[74] and even for distinguishing between the subspecies *lactis* and *cremoris* of *Lc. lactis*.[76] 16S rRNA sequence data from LAB have been accumulated during recent years, and the list of available probes published by Pot et al.[135] has of course expanded considerably since then. For certain applications (e.g., analysis of food samples) it may also be interesting to determine the occurrence of specific groups of LAB. Genus- and group-specific probes have been developed for such purposes.[13,59,82]

Bacteria typically have five to seven copies of each rRNA gene in the chromosome. This has been exploited in a restriction fragment length polymorphism (RFLP) molecular typing method, more commonly known as ribotyping.[136] This method appears useful for species and subspecies recognition in some cases, e.g., lactococci,[79] *Lb. plantarum*,[137] and pediococci,[100] but not in others, e.g., *Lb. reuteri*.[138] It is interesting to note that *Lb. reuteri*, a quite homogeneous species phenotypically, showed heterogeneous ribopatterns, while *Lb. plantarum*, phenotypically heterogeneous, had more homogeneous ribopatterns.[137] This method, therefore, has to be evaluated from case to case as to its applicability in strain or species recognition. Automated instruments that generate large databases of reproducible ribopatterns are available,[139] possibly setting the standard for interlaboratory work with this identification method.

The PCR technique is becoming more and more useful for classification purposes. With this technique it is possible to amplify a gene or a part of a gene from a very limited number of cells for subsequent DNA sequencing. One of the targets for such an amplification are obviously rRNA genes, and this method has replaced the reverse transcriptase technique for collecting rRNA sequence data for phylogenetic analysis. With automated sequencing systems and convenient direct PCR sequencing methods, it has become an easy task to determine the 16S rRNA sequence from any bacterium in a short time. PCR can also be used in combination with probing techniques[78,82,134] or actually replace them since the oligonucleotide probes designed from 16S rRNA sequencing also can be used in PCR applications.

A number of fingerprinting techniques based on PCR have also been developed. The most commonly known is randomly amplified polymorphic DNA (RAPD).[140,141] RAPD has been shown to be applicable for distinguishing strains belonging to the so-called *Lb. acidophilus* group, which contains six species with similar phenotype that are considered important as candidates for probiotic strains,[142,143] although several methods can be used for this particular cluster of species (see below). In a study of several *Lb. plantarum* strains, RAPD was found to be more suitable to discriminate between different subtypes of species than ribotyping.[144] The simplicity of this method makes it very attractive, but reproducible results require highly standardized conditions. The method of breaking the cells to obtain a crude DNA preparation is one of the critical steps in this regard.[144] Another fingerprinting PCR-based method with similarities to RAPD is REP-PCR (with variants known as ERIC-PCR and BOX-PCR), which exploits conserved repetitive DNA sequences in bacterial genomes.[145] Reproducibility is claimed to be higher than for RAPD, and successful use for LAB has been reported.[146–148] Intergenic spacer sequence polymorphism in variants has also been used in PCR-based methods (tDNA-PCR, ISR-PCR) for species identification in LAB.[123,149–151]

Other genotypic fingerprinting methods are based on restriction endonuclease cleaving of the chromosomal DNA. When rare-cutting enzymes (e.g., *Not*I) are used, creating large and relatively few fragments, pulse field gel electrophoresis (PFGE) is used to separate the fragments, and the method is often referred to as just PFGE. PFGE is considered the gold standard in classifying bacterial strains with genotypic fingerprinting at the strain level because of its high discriminatory power. This has been shown also for LAB.[152–154] PFGE is therefore used as the principal method for creating unique "fingerprints" of defined commercial strains by vendors for starter cultures and/or probiotics. Using enzymes that cut more often complicates the analysis (many more fragments) and requires sophisticated statistical analysis, but the digests can be run on conventional gels, although highly standardized. The method is often referred to as restriction endonuclease analysis

(REA) and has been successfully used for classifying LAB with very high discriminatory power.[155–157] Amplified fragment length polymorphism (AFLP) is a PCR-based REA in which a selected portion of the fragments are amplified, visualized, and the banding pattern analyzed as for other fingerprinting methods.[158] The method has good resolution at the strain level and has the potential to replace PFGE as a standard fingerprinting method. The method was considered very useful in classifying LAB of the *Lb. acidophilus* group.[143]

In several studies the genetic methods described above have been compared in classifying LAB. One of the most extensive and informative studies is still the collective work done by M. Ståhl, M.-L. Johansson, and coworkers. They used a set of *Lb. reuteri* and *Lb. plantarum* strains and systematically characterized these with phenotypic tests, DNA-DNA homology, REA, automated PCR sequencing of rRNA, ribotyping, and RAPD.[137,138,144,155–157,159] This work elegantly shows that each method has advantages and disadvantages and that one single method is not the solution for all applications, but rather that the methods complement each other.

Another technique that has proven to be very useful in the classification of LAB is soluble protein patterns. The technique involves polyacrylamide gel electrophoresis of whole cell proteins and statistical analyses of the patterns obtained (for reviews, see Refs.[160] and [161]. A database of digitized and normalized patterns from a large number of LAB was constructed,[135] and the similarity clusters clearly correlate with results based on genetic data, i.e., rRNA sequences and DNA-DNA homologies.[135,160,162] The method can be used directly as a screening method to assign a particular strain to a species when the pattern is compared with those in the database. As for other methods involving a statistical analysis of banding patterns, standardization is the key to reproducible results. In contrast to methods analyzing patterns derived from DNA, growth conditions are extremely important since the protein composition of the cells may vary depending on media, temperature, etc. Therefore, interlaboratory comparisons of patterns may not be reliable.[161]

To summarize, a number of alternatives to classical phenotypic/biochemical identification of LAB have emerged since 1990. Which one to use is often a matter of taste, probably depending on what methods a particular laboratory starts to use. Each method has its advantages and disadvantages. For thorough identification/classification in bacterial systematics, it is still recommended to apply a polyphasic approach,[163] i.e., using several phenotypic, chemotaxonomic, and genotypic methods.

IV. METABOLISM OF LACTIC ACID BACTERIA

The essential feature of LAB metabolism is efficient carbohydrate fermentation coupled to substrate-level phosphorylation. The generated ATP is subsequently used for biosynthetic purposes. LAB as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product is, of course, lactic acid (>50% of sugar carbon). It is clear, however, that LAB adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end product patterns. This section will describe the well-known fermentation pathways and how various sugars are fermented. It will also show some of the more unusual features of LAB metabolism, which may be of importance in their natural habitat.

A. Major Fermentation Pathways

1. Hexose Fermentation

As mentioned in the classification section in this chapter, there are two major pathways for hexose (e.g., glucose) fermentation within LAB (Fig. 2). The transport and phosphorylation of glucose may occur as outlined, i.e., transport of free sugar and phosphorylation by an ATP-dependent glucokinase. Some species use the phosphoenolpyruvate : sugar phosphotransferase system (PTS), in which phosphoenolpyruvate is the phosphoryl donor (see Sec. V.B). In either case, a high-energy phosphate bond is required for activation of the sugar.

Glycolysis (Embden-Meyerhof-Parnas pathway), used by all LAB except leuconostocs, group III lactobacilli, oenococci, and weissellas, is characterized by the formation of fructose-1,6-diphosphate (FDP), which is split by a FDP aldolase into dihydroxyacetonephosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). GAP (and DHAP via GAP) is then converted to pyruvate in a metabolic sequence including substrate-level phosphorylation at two sites. Under normal conditions, i.e., excess sugar and limited access to oxygen, pyruvate is reduced to lactic acid by a NAD^+-dependent lactate dehydrogenase (nLDH), thereby reoxidizing the NADH formed during the earlier glycolytic steps. A redox balance is thus obtained, lactic acid is virtually the only end product, and the metabolism is referred to as a homolactic fermentation.

The other main fermentation pathway has had several designations, such as the pentose phosphate pathway, the pentose phosphoketolase pathway, the hexose monophosphate shunt and, used by Kandler and Weiss[113] in *Bergey's Manual*, the 6-phosphogluconate pathway. I will refer to it as the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway, thereby recognizing a key step in the metabolic sequence (the phosphoketolase split) and at the same time distinguishing it from the bifidum pathway, which also involves phosphoketolase but does not have 6-phosphogluconate as an intermediate.[164] It is characterized by initial dehydrogenation steps with the formation of 6-phosphogluconate, followed by decarboxylation. The remaining pentose-5-phosphate is split by phosphoketolase into GAP and acetyl phosphate. GAP is metabolized in the same way as for the glycolytic pathway, resulting in lactic acid formation. When no additional electron acceptor is available (see Sec. IV.D), acetyl phosphate is reduced to ethanol via acetyl CoA and acetaldehyde. Since this metabolism leads to significant amounts of other end products (CO_2, ethanol) in addition to lactic acid, it is referred to as a heterolactic fermentation.

The terminology regarding these pathways and the bacteria that use them is rather confusing, and it is perhaps appropriate to add a note of caution. In general, the term "homofermentative LAB" refers to those in the group that use the glycolytic pathway for glucose fermentation, whereas "heterofermentative LAB" are those that use the 6-PG/PK pathway. However, it should be noted that glycolysis may lead to a heterolactic fermentation (meaning significant amounts of end products other than lactic acid) under certain conditions and that some LAB regarded as homofermentative use the 6-PG/PK pathway when metabolizing certain substrates. This will be discussed in more detail later.

In theory, homolactic fermentation of glucose results in 2 mol of lactic acid and a net gain of 2 ATP per mol glucose consumed. Heterolactic fermentation of glucose through the 6-PG/PK pathway gives 1 mol each of lactic acid, ethanol, and CO_2 and 1 mol ATP/mol glucose. In practice, these theoretical values are seldom obtained. A conversion factor of 0.9 from sugar to end-product carbon is common and probably reflects an

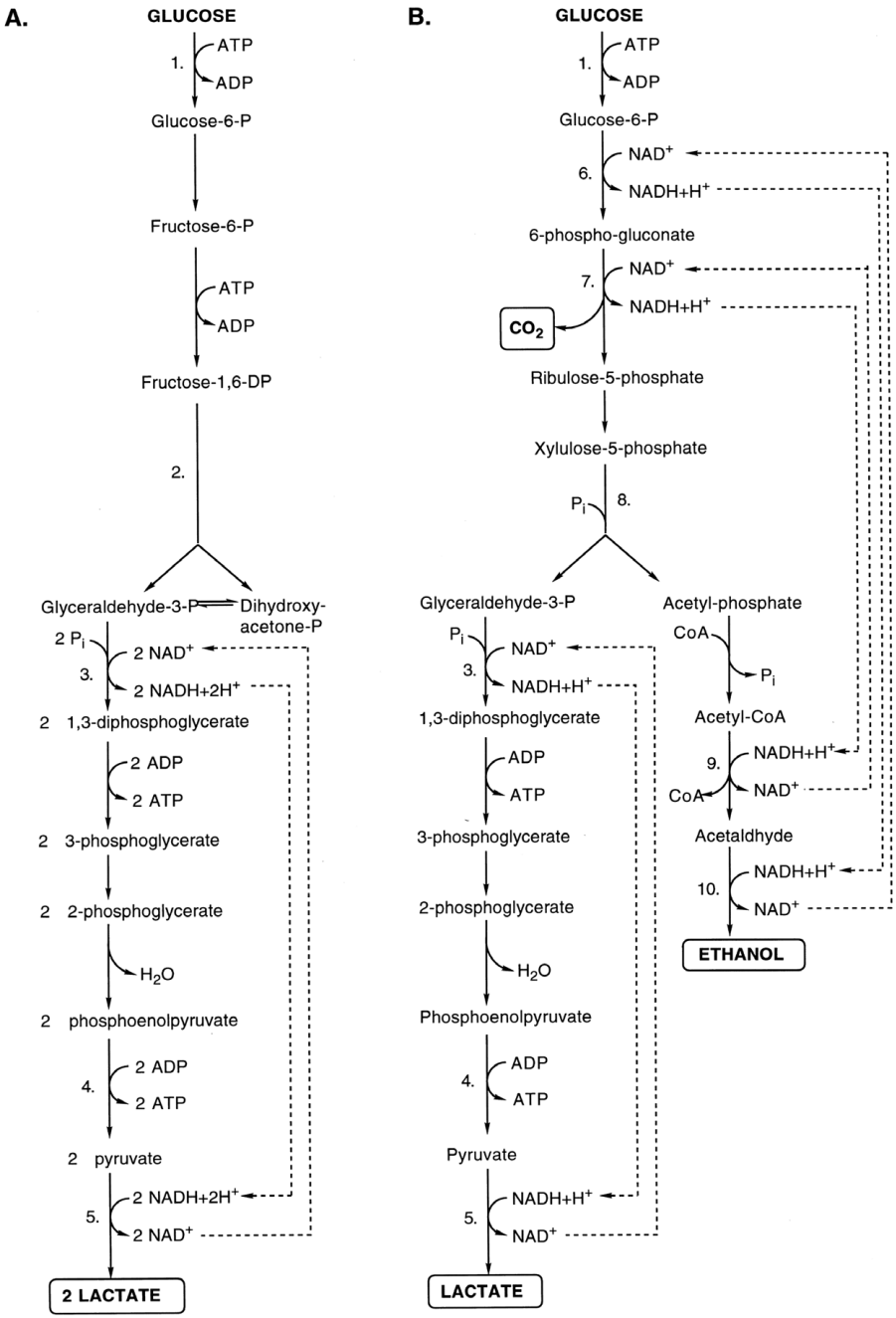

Figure 2 Major fermentation pathways of glucose: (A) homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway); (B) heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered: 1. Glucokinase; 2. fructose-1,6-diphosphate aldolase; 3. glyceradehyde-3-phosphate dehydrogenase; 4. pyruvate kinase; 5. lactate dehydrogenase; 6. glucose-6-phosphate dehydrogenase; 7. 6-phosphogluconate dehydrogenase; 8. phosphoketolase; 9. acetaldehyde dehydrogenase; 10. alcohol dehydrogenase.

incorporation of sugar carbon into the biomass, even though most growth factors (e.g., amino acids, nucleotides, and vitamins) are supplied in excess in the rich media frequently used. These complex media may also contribute to other fermentation balances and to the formation of other end products, in particular acetic acid, since compounds like organic acids, amino acids, and sugar residues can alter the fermentation.[115] The presence of oxygen may also have a significant effect on the metabolism (see Ref. 165 and Sec. IV.C).

Hexoses other than glucose, such as mannose, galactose, and fructose, are fermented by many LAB. The sugars enter the major pathways at the level of glucose-6-phosphate or fructose-6-phosphate after isomerization and/or phosphorylation. One important exception is galactose metabolism in LAB, which uses a PTS for uptake of this sugar, e.g., *Lc. lactis*, *E. faecalis*, and *Lb. casei.* In these species, the galactose-6-phosphate formed by the PTS is metabolized through the tagatose-6-phosphate pathway[166] (Fig. 3A). Tagatose is a stereoisomer of fructose, but separate enzymes are required for the metabolism of the tagatose derivatives. The tagatose pathway coincides with

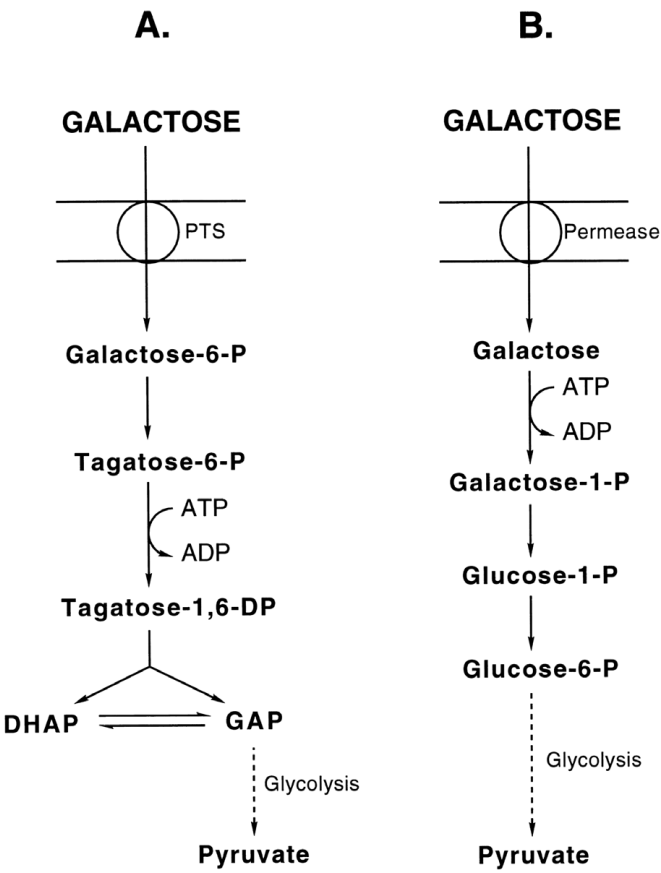

Figure 3 Galactose metabolism in lactic acid bacteria: (A) tagatose-6-phosphate pathway; (B) Leloir pathway.

glycolysis at the level of GAP. Many strains in this category also have the capacity to transport galactose with a permease and convert it to glucose-6-phosphate via the Leloir pathway (Fig. 3B).[167] This pathway is also used by galactose-fermenting LAB that transport galactose with a permease and lack a galactose PTS.[22,98,115]

2. Disaccharide Fermentation

Depending on the mode of transport, disaccharides enter the cell either as free sugars or as sugar phosphates. In the former case, the free disaccharides are split by specific hydrolases to monosaccharides, which then enter the major pathways described above. In the latter case, i.e., when sugar PTS are involved, specific phosphohydrolases split the disaccharide phosphates into one part free monosaccharides and one part monosaccharide phosphates.

By far the most studied disaccharide metabolism in LAB is lactose fermentation. Most strains of *Lc. lactis*, at least those used as dairy starters, contain a lactose PTS[168,169] A lactose PTS from *Lb. casei* is also well characterized.[170] In these strains, lactose enters the cytoplasm as lactose phosphate, which is cleaved by phospho-β-D-galactosidase (P-β-gal) to yield glucose and galactose-6-phosphate. Glucose is phosphorylated by glucokinase and metabolized through the glycolytic pathway, whereas galactose-6-phosphate is metabolized through the tagatose-6-phosphate pathway. The enzyme system of the lactose PTS and P-β-gal are generally inducible and repressed by glucose.[115]

The lactose metabolism of *Lc. lactis* is one of the most well-studied systems of sugar fermentation occurring in LAB. This knowledge is relevant for the understanding of sugar metabolism in general[171] and of the processes of milk fermentation in particular. However, an equally common way to metabolize lactose among LAB is by means of a lactose carrier (permease) and subsequent cleavage by β-galactosidase (β-gal) to yield glucose and galactose,[98,172,173] which may then enter the major pathways. Some reports on this subject would suggest that many LAB contain both a lactose PTS and a lactose permease system for lactose metabolism, since both P-β-gal and β-gal activity were found in the same strains.[172,174] However, low P-β-gal activity in strains with high β-gal activity may represent an artefact, since the artificial substrate used for P-β-gal (ortho-nitrophenylgalactose phosphate) may be hydrolyzed by β-gal or by a phosphatase yielding the substrate for β-gal.[98,174]

Some of the thermophilic LAB, e.g., *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. acidophilus*, only metabolize the glucose moiety after transport of lactose and cleavage by β-gal, while galactose is excreted into the medium.[174,175] Lactose transport and metabolism in the economically important species *S. thermophilus* has also been thoroughly studied.[176] Galactose excretion has been attributed to a low galactokinase activity[174,177] but is also energetically favorable and a feature of the lactose transporter.[176]

Maltose fermentation among LAB has been studied most extensively in lactococci. A permease system for transport seems to be operational.[22,178] An interesting feature of maltose metabolism in *Lc. lactis* 65.1 is that maltose is cleaved by a maltose phosphorylase into glucose and β-glucose-1-phosphate. Only the glucose moiety is used in glycolysis, whereas β-glucose-1-phosphate probably is a precursor for cell wall synthesis.[178]

Sucrose fermentation mediated by a permease system is initiated by the cleavage of the sugar by sucrose hydrolase to yield glucose and fructose, which enter the major pathways. In some lactococci, sucrose is transported by sucrose PTS and a specific

sucrose-6-phosphate hydrolase cleaves the sucrose-6-phosophate to glucose-6-phosphate and fructose.[179] The sucrose PTS and sucrose-6-phosphate hydrolase are induced by the presence of sucrose in the medium.[179] Sucrose may also act as a donor of monosaccharides for exopolysaccharide formation in certain LAB. In dextran production by *Ln. mesenteroides*, sucrose is cleaved by a cell wall–associated enzyme, dextransucrase. The glucose moiety is used for dextran synthesis and fructose is fermented in the usual manner.[180]

Fermentation of other disaccharides, such as cellobiose, melibiose, and trehalose, has not been studied to any large extent. The ability to ferment these sugars differ between different species of LAB. Presumably, the metabolism is mediated by specific transport systems and hydrolases, resulting in the respective monosaccharides (or monosaccharide phosphates), which enter the common pathways.

3. Fermentation of Pentoses and Related Compounds

Pentoses are readily fermented by many LAB. In general, specific permeases are used to transport the sugars into the cells. Inside, the pentoses are phosphorylated and converted to ribulose-5-phosphate or xylulose-5-phosphate by epimerases or isomerases.[115] These compounds can then be metabolized by the lower half of the 6-PG/PK pathway (Fig. 2B). This would imply that only heterofermentative LAB can utilize pentoses, but this is not the case. In fact, disregarding some strain and species differences, all genera of LAB are pentose positive with one exception, the group I lactobacilli. Homofermentative LAB that utilize pentoses generally do so in the same way as heterofermentative LAB. The phosphoketolase of these species is induced by substrates fermented by the 6-PG/PK pathway and repressed by glucose.[115,181] The heterolactic fermentation of pentoses results in a different end product pattern compared to glucose fermentation. No CO_2 is formed, and since no dehydrogenation steps are necessary to reach the intermediate xylulose-5-phosphate, the reduction of acetyl phosphate to ethanol becomes redundant. Instead, acetyl phosphate is used by the enzyme acetate kinase in a substrate-level phosphorylation step yielding acetate and ATP. Fermentation of pentoses thus leads to production of equimolar amounts of lactic acid and acetic acid.

Although gluconate is not a pentose, this is an appropriate place to mention gluconate fermentation, which can be performed by some LAB. Like pentoses, gluconate is fermented by the 6-PG/PK pathway, yielding a heterolactic fermentation by species regarded as homofermentative. The metabolism has been studied in *E. faecalis*, but a similar pathway may also exist in *Lb. casei*.[182] In *E. faecalis*, gluconate is transported by an inducible gluconate-PTS and the resulting 6-phosphogluconate enters the 6-PG/PK pathway.[182] Many group II and group III lactobacilli also ferment gluconate,[113] presumably in a metabolic sequence similar to that for *E. faecalis*, although transport may be mediated by a permease in some cases. Since a dehydrogenation step is necessary before the phosphoketolase reaction, some acetyl phosphate has to be reduced to ethanol in order to maintain the redox balance.

A few species of LAB, e.g., *Lb. casei*, can grow at the expense of pentitols. These are translocated through the membrane by specific pentitol PTS. The resulting pentitol phosphates are oxidized to pentose phosphates by dehydrogenases and subsequently metabolized through the 6-PG/PK pathway.[182] Similar to gluconate fermentation, some ethanol is produced from acetyl phosphate because of the need to reoxidize the NADH formed during the initial dehydrogenation.

4. Sugar Fermentation and Metabolic Categories of LAB

From the descriptions of sugar fermentation patterns of LAB presented above, it is possible to divide the group broadly into three metabolic categories. The genus *Lactobacillus* contains species that can be placed in all three categories, and this is in fact the basis for the division of the genus into three groups.[114] The first category includes the group I lactobacilli and some individual species from other genera, which are obligately homofermentative, meaning that sugars only can be fermented by glycolysis. The second category includes leuconostocs, group III lactobacilli, oenococci, and weissellas, which are obligately heterofermentative, meaning that only the 6-PG/PK pathway is available for sugar fermentation. The apparent difference on an enzyme level between these two categories is the presence or absence of the key enzymes of glycolysis and 6-PG/PK pathway, FDP aldolase and phosphoketolase, respectively. Obligately homofermentative species possess a constitutive FDP aldolase and lack phosphoketolase, whereas the opposite holds for obligately heterofermentative species.[113,115] This leads to the obvious difference in end product formation from glucose, but also to the inability of the group I lactobacilli to attack pentoses (and gluconate). The third category, including the remaining LAB (i.e., group II lactobacilli and most species of enterococci, lactococci, pediococci, streptococci, tetragenococci, and vagococci), holds an intermediate position. They resemble the obligately homofermentative LAB in that they possess a constitutive FDP aldolase, resulting in the use of glycolysis for hexose fermentation. As mentioned previously, pentoses (and presumably gluconate and pentitols when fermented) induce the synthesis of phosphoketolase, resulting in a heterolactic fermentation. These LAB are thus homofermentative with regard to hexoses and heterofermentative with regard to pentoses and some other substrates and should, therefore, be termed facultatively heterofermentative.[113,115]

The position of species in the genus *Carnobacterium* is somewhat unclear. These bacteria were first classified as heterofermentative (then under the designation *Lb. divergens*), since gas and acetic acid were produced in significant amounts.[113] Later, more detailed studies showed that glucose is fermented almost entirely to lactic acid, probably by the glycolytic pathway, the other products arising from metabolism of components in the medium other than glucose and/or some deviation of the pyruvate formed by glycolysis.[120] Since carnobacteria generally ferment ribose and gluconate,[28,113] they should probably be regarded as facultatively heterofermentative.

Recent reports suggest that there exists some deviation from the normal patterns described above. Some lactobacilli regarded as obligately heterofermentative may in fact possess the apparent missing key enzyme. A strain of *Lb. brevis* (group III) was shown to ferment fructose by the glycolytic pathway including a fructose-inducible FDP aldolase.[183] The fermentation of a pentose by *Lc. lactis* IO-1 was shown to result almost exclusively in lactic acid under certain conditions.[184] It was suggested that transaldolases and transketolases of the classical pentose phosphate pathway were involved in converting the substrate for normal glycolysis. Whether these examples represent some exceptional cases or something more general is not clear at the moment. At any rate, the borders between the metabolic categories of LAB may not be as absolute as previously thought.

5. Configuration of Lactic Acid

During the fermentation of sugars, different species of LAB produce either exclusively L-lactic acid, exclusively D-lactic acid, approximately equal amounts of both, or predominantly one form but measurable amounts of the other.[39,113,185] This depends on the

presence of specific NAD^+-dependent lactate dehydrogenases (nLDH) and their respective activities. Thus, if both D- and L-lactic acid are formed, there are generally one D-nLDH and one L-nLDH present. Only a few species, e.g., *Lb. curvatus* and *Lb. sakei*, produce an enzyme, termed a racemase, which converts L-lactic acid to D-lactic acid.[185] In this case, the L-lactic acid initially produced induces the racemase, which results in a mixture of D- and L-lactic acid. The reason for or possible advantage to producing a mixture of D- and L-lactic acid is not clear. Inctivation of L-nLDH by mutation in a strain of *Lb. plantarum* did not change the growth rate in laboratory media or the global lactic acid concentration at any stage of the culture. The absence of L-lactic acid was simply accompanied by an increase in D-lactic acid production,[186] indicating that the reduction of pyruvate to lactate is not a rate-limiting step in glycolysis. Worthy of note in this regard is the species *Lb. bavaricus*, which genetically appears to be a variant of *Lb. sakei* with no racemase activity and therefore only produces L-lactic acid[187] *P. pentosaceus* and many lactobacilli change the ratio of the isomers during batch growth. Generally, L-lactic acid is the major form produced in the early growth phase and D-lactic acid in the late to stationary phase.[185] The pH and internal pyruvate concentration have been thought to influence the activities of the LDH and thus the ratio of the isomers at different growth phases,[185] but not much is known in this regard. *Lb. casei* and lactococci possess an allosteric L-nLDH, which is activated by FDP (see Sec. IV.B). Enterococci and streptococci also possess this type of LDH.[185]

B. Fates of Pyruvate

It is well known that LAB may change their metabolism in response to various conditions, resulting in a different end-product pattern than seen with glucose fermentation under normal conditions. In most of these cases, the change can be attributed to an altered pyruvate metabolism, the use of external electron acceptors, or both, as these may be connected to each other. This will be discussed below.

The essential feature of most bacterial fermentations is the oxidation of a substrate to generate energy-rich intermediates, which subsequently can be used for ATP production by substrate-level phosphorylation. The oxidation results in the formation of NADH from NAD^+, which has to be regenerated in order for the cells to continue the fermentation. Pyruvate holds a key position in many fermentations in serving as an electron (or hydrogen) acceptor for this regeneration step. Indeed, this is true in both major fermentation pathways used by LAB (Fig. 2). Under certain circumstances, LAB use alternative ways of utilizing pyruvate than the reduction to lactic acid. The alternative fates of pyruvate are depicted in Fig. 4. Not all of these reactions are used by a single strain, but they represent a summary of the LAB group as a whole.[115] Different species may use different pathways, depending on conditions and enzymatic capacity. Some of these reactions may be operational even under normal glucose fermentation, but then serving an anabolic role. For instance, the formation of acetyl CoA can be required for lipid biosynthesis.[188]

1. The Diacetyl/Acetoin Pathway

The pathway(s) leading to diacetyl (butter aroma) and acetoin/2,3-butanediol (Fig. 4) is common among LAB[115] and is very significant technologically in the fermentation of milk.[168,189] However, this metabolism proceeds to a significant degree only if there is a pyruvate surplus in the cell relative to the need for NAD^+ regeneration. A pyruvate surplus can be created in two ways: (a) a source of pyruvate other than the fermented

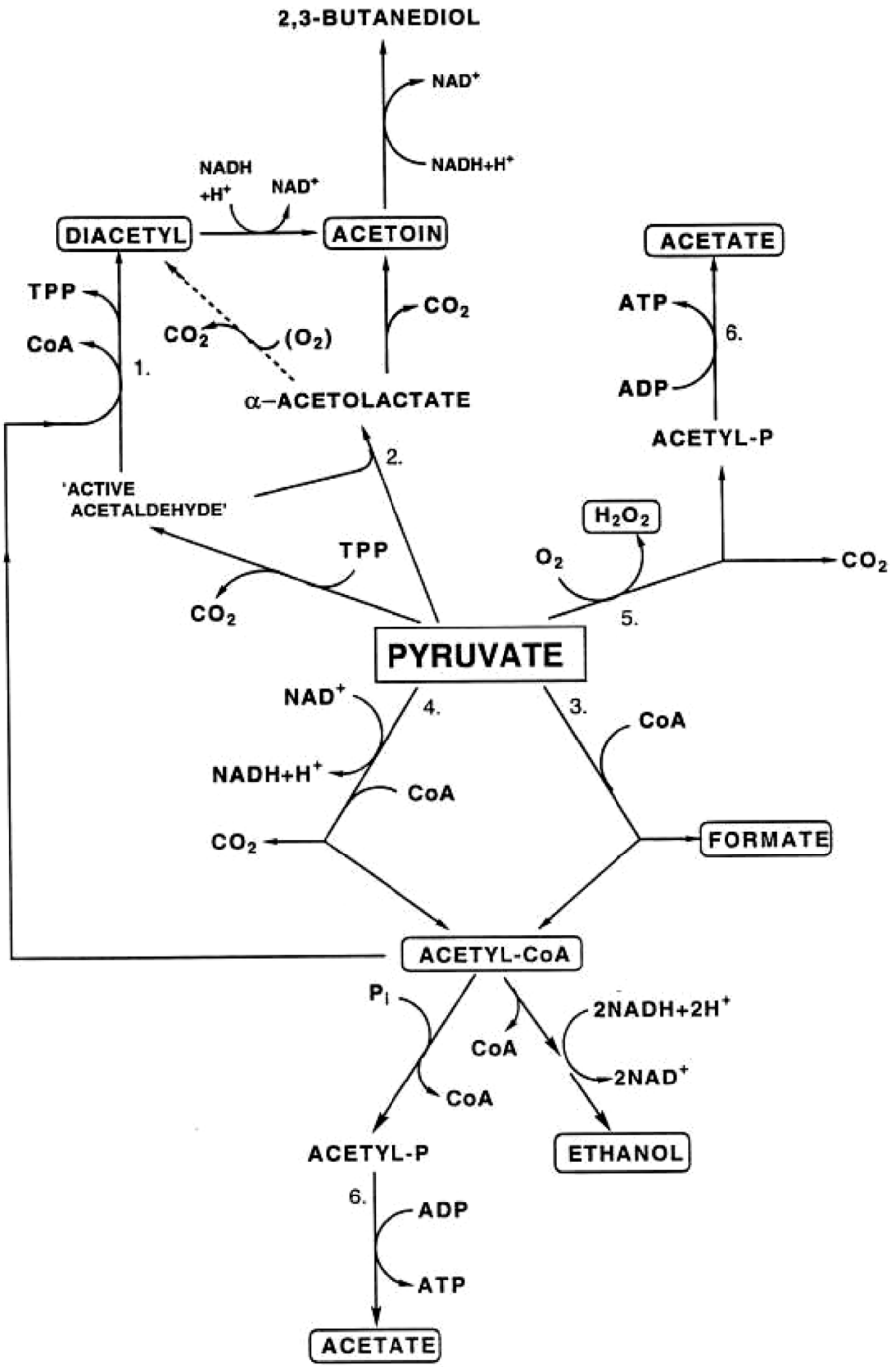

Figure 4 Pathways for the alternative fates of pyruvate. Dashed arrow denotes a nonenzymatic reaction. Important metabolites and end products are framed. Selected enzymatic reactions are numbered: 1. diacetyl synthase; 2. acetolactate synthase; 3. pyruvate-formate lyase; 4. pyruvate dehydrogenase; 5. pyruvate oxidase; 6. acetate kinase.

carbohydrate exists in the growth medium and (b) another compound acts as electron acceptor, thus sparing the pyruvate formed by carbohydrate fermentation. The former is what occurs in milk, where additional pyruvate originates from the breakdown of citrate, which is present in significant amounts (~1.5 mg/mL). This topic has been studied extensively, and a review by Hugenholtz[190] covers most of the aspects of citrate metabolism in connection with diacetyl/acetoin production in LAB. Citrate is transported into the cell by a citrate permease and cleaved by citrate lyase to yield oxaloacetate and acetate. Oxaloactetate is further decarboxylated to pyruvate and CO_2 by oxaloacetate decarboxylase. Species used in the dairy industry for the purpose of diacetyl production are *Lc. lactis* subsp. *lactis* (biovar. *diacetylactis*) and *Ln. mesenteroides* subsp. *cremoris* (former name *Ln. cremoris* or *Ln. citrovorum*). These species may vary somewhat in induction pattern and pH optimum of the enzymes of citrate dissimilation and diacetyl/acetoin production.[189,191–193] In general, low sugar concentrations and low pH favor diacetyl/acetoin formation. Historically, there was some debate as to which of the two possible pathways (Fig. 4) was the most important. Evidence now clearly favors the route via α-acetolactate since α-acetolactate can be detected as an intermediate in cultures producing diacetyl and an α-acetolactate synthase has been identified in several LAB.[190] Diacetyl is formed by chemical decomposition of α-acetolactate (nonenzymatic). This reaction is favored by aeration and low pH. Acetoin and/or 2,3-butanediol is produced in much larger amounts than diacetyl, but does not contribute to the aroma.[193] The extensive knowledge obtained in recent years of this system, both biochemically and genetically, is now being exploited in metabolic engineering strategies to control diacetyl production.[14,15,190,194] Interestingly, one of the approaches was to create a pyruvate surplus according to the second possibility, as mentioned above. By overexpressing NADH oxidase, oxygen could efficiently act as an electron acceptor and the surplus pyruvate could subsequently be diverted to diacetyl by several strategies.[15]

2. The Pyruvate-Formate Lyase System

Another "branch" of pyruvate metabolism, shown in Fig. 4, consists of the pyruvate-formate lyase system. The enzyme pyruvate-formate lyase catalyzes the reaction of pyruvate and CoA to formate and acetyl CoA. Acetyl CoA may be used either as an electron acceptor, resulting ultimately in ethanol formation, as a precursor for substrate-level phosphorylation via acetyl phosphate, or both. The system has been shown to be operational in several species of LAB.[115] Most notably, the pathway is used by some strains of *Lb. casei* and *Lc. lactis*, grown in anaerobic continous culture under substrate limitation, resulting in a change from a homolactic to a heterolactic, in this case mixed acid, fermentation.[195,196] End products formed are lactate, acetate, formate, and ethanol. Larger amounts of the products of the pyruvate-formate lyase system (acetate, formate, and ethanol) are formed with decreasing growth rate, i.e., with lowering of the dilution rate in the continuous culture system.[196] A similar end product pattern is found when some strains of *Lc. lactis* ferment galactose[167] or maltose.[178]

It has been proposed that the change to a mixed acid fermentation by these strains is due to a reduction of the glycolytic rate. This affects the levels of glycolytic intermediates and subsequently the activities of the enzymes that compete for pyruvate, nLDH, and pyruvate-formate lyase. The nLDH:s of *Lc. lactis* and *Lb. casei* are allosteric enzymes with a specific requirement for the key glycolytic intermediate FDP (see Fig. 2) for activity.[185] In general, lower intracellular levels of FDP are found in cells of *Lc. lactis* performing mixed acid fermentation compared to those found in cells during homolactic

fermentation.[167,178,196] Similarily, the levels of triose phosphates, known inhibitors of pyruvate-formate lyase,[197] are also lower in heterolactic cells.[167] Thus, during a semi-starved state, caused by either substrate limitation or the nature of the substrate, the cells respond by regulating certain enzyme activities so as to partly prevent pyruvate from being reduced to lactic acid. Instead, energy can be gained by using the pyruvate-formate lyase pathway, since there is a substrate-level phosphorylation site involving acetyl phosphate and acetate kinase (Fig. 4). Indeed, increased molar growth yield for glucose (Y_{glc}) was obtained in the continuous culture system at low compared to high growth rates,[196] indicating that more ATP/glucose was formed during mixed acid fermentation. Recently, the role of FDP in the shift to heterolactic fermentation has been questioned and the effect of the $NADH/NAD^+$ ratio on glyceraldehyde-3-phosphate dehydrogenase and nLDH has been suggested as the main factor.[198,199] FDP could nevertheless have an indirect role by stimulating components involved in global control of carbon metabolism (see Sec. VI).

The pyruvate-formate lyase system is only active anaerobically, which is consistent with the enzyme being extremely oxygen sensitive[197,200] and presumably inactivated when cells are exposed to air.

3. The Pyruvate Oxidase Pathway

Oxygen has a profound effect on the fate of pyruvate in LAB. This effect may be direct, mediated by the enzyme pyruvate oxidase (Fig. 4), or indirect through reactions of oxygen with the flavin-containing enzymes NADH : H_2O_2 oxidase and NADH : H_2O oxidase.[165] Pyruvate oxidase converts pyruvate to CO_2 and acetyl phosphate with the formation of H_2O_2 (Fig. 4). The enzyme has been suggested to be involved in aerobic metabolism of *Lb. plantarum*, which forms significant amounts of acetic acid aerobically.[201] In the study by Sedewitz et al.,[201] pyruvate oxidase had the highest activity in the early stationary phase of growth and lactose-grown cells had generally higher levels of the enzyme than glucose-grown cells. Since this strain of *Lb. plantarum* had much lower growth rate on lactose than on glucose, a possible explanation of the elevated pyruvate oxidase level could be that the levels of glycolytic intermediates in some way regulated enzyme synthesis.[201] A certain similarity to the regulation of the pyruvate-lyase system in *Lc. lactis*, described above, can thus be noted.

4. The Pyruvate Dehydrogenase Pathway

Evidence have been obtained that a pyruvate dehydrogenase enzyme complex is active in lactococci.[188,202,203] The enzyme complex produces acetyl CoA (Fig. 4) and thus resembles the pyruvate-formate lyase system. The study by Cogan et al.[188] indicated that pyruvate dehydrogenase has an anabolic role in producing acetyl CoA for lipid synthesis under aerobic conditions. Anaerobically, this role is probably played by pyruvate-formate lyase, which has the advantage of not reducing NAD^+ in the process. Exposed to air, the cells are dependent on a functional pyruvate dehydrogenase for acetyl CoA production, since pyruvate-formate lyase is inactivated by oxygen. The excess NADH formed can be reoxidized by NADH oxidases. Similar to the pyruvate-formate lyase system, the pyruvate dehydrogenase can also play a role in catabolism, but then primarily under aerobic conditions. The effect can be rather dramatic, however. Aerated cultures of nongrowing cells of *Lc. lactis* can perform a homoacetic fermentation under substrate limitation.[203] In this case, all the pyruvate generated from glycolysis is channeled through the pyruvate dehydrogenase complex with acetic acid (and presumably CO_2) as

the final product. Under these conditions, the nLDH has probably very low activity and cannot effectively compete for pyruvate. A prerequisite for this metabolism to occur is that the NADH formed during both glycolysis and in the pyruvate dehydrogenase reaction can be reoxidized by NADH oxidases.[203]

C. Oxygen as Electron Acceptor

In reactions with NADH oxidases, oxygen acts as an external electron acceptor. In many cases this can be advantageous to LAB.

NADH oxidases seem to be widespread among LAB, and the systems are often induced by oxygen. The products of the reactions are either NAD^+ and H_2O_2 or NAD^+ and H_2O, depending on whether the enzyme mediates a two- or four-electron transfer. Most LAB also possess a NADH peroxidase, which use H_2O_2 as electron acceptor with the formation of H_2O. This reaction may mask initial NADH : H_2O_2 oxidase activity.[203] NADH oxidases may compete efficiently with nLDH in homofermentative LAB. This may create a situation similar to the breakdown of citrate described earlier, i.e., a pyruvate surplus available for metabolism through the diacetyl/acetoin pathway. Increased production of acetoin in aerated cultures, compared to unaerated, has been shown for *Lc. lactis*,[188] homofermentative lactobacilli, and carnobacteria.[204] Worthy of note is that heterofermentative LAB (leuconostocs and group III lactobacilli) did not respond to aeration with acetoin production, whereas most of the homofermentative LAB did.[204] The probable explanation is that in heterofermentative LAB, the NADH oxidases do not compete with nLDH, but rather with acetaldehyde dehydrogenase and alcohol dehydrogenase, the enzymes of the ethanol "branch" of the 6-PG/PK pathway (Fig. 2 and see below). Accordingly, a pyruvate surplus is not created and lactic acid remains the main product of pyruvate metabolism.

LAB that ferment glucose by the 6-PG/PK pathway use acetyl phosphate (or more accurately, acetyl CoA) as an electron acceptor in addition to pyruvate. Reduction of acetyl phosphate (via acetyl CoA) constitutes the ethanol branch of the pathway. This route is in a way a waste of acetyl phosphate, since this compound can be used in substrate-level phosphorylation with the production of ATP. Heterofermentative LAB may circumvent this waste by using external electron acceptors. As was hinted above, oxygen can play an active role. Lucey and Condon[205] showed that strains of *Leuconostoc* sp. doubled the Y_{glc} in aerated cultures compared to unaerated. In addition, the growth rate was higher with aeration. The effect was attributed to an active NADH oxidase, which efficiently prevented ethanol formation. Instead, acetate was formed from acetyl phosphate, additional ATP could be produced (by the acetate kinase reaction) and as a consequence Y_{glc} increased. A NADH oxidase–deficient mutant did not shift from ethanol to acetate production and Y_{glc} was the same aerobically and anaerobically, thus supporting the role of NADH oxidase in aerobic metabolism. The shut-off of the ethanol branch of the 6-PG/PK pathway in the presence of oxygen seems to be very common among heterofermentative LAB. However, an increase of Y_{glc} is not always seen.[204] The reason for this is not clear.

The active role of oxygen as electron acceptor in the metabolism of LAB is further illustrated by the fact that certain substrates are fermented only when oxygen is available. This is especially true for the fermentation of reduced compounds, such as polyols. Examples are oxygen-dependent glycerol fermentation by *P. pentosaceus*[206] and mannitol fermentation by *Lb. casei*.[207] It is also well known that some heterofermentative lactobacilli, most notably strains of *Lb. brevis*, are almost unable to ferment glucose

anaerobically, but will ferment it aerobically.[208] This is due to a deficiency in the ethanol branch of the 6-PG/PK pathway, more specifically a lack of acetaldehyde dehydrogenase.[209] The deficiency creates an absolute requirement for an external electron acceptor (e.g., oxygen) in the fermentation of glucose.

Even lactate, the end product of "normal" metabolism, can be fermented to acetate and CO_2 by some LAB.[210,211] A pathway for this oxygen-dependent lactate fermentation of *Lb. plantarum* has been proposed, involving NAD^+-dependent and/or NAD^+-independent LDH, pyruvate oxidase, and acetate kinase.[210] The ATP yield would be 1 ATP per mol lactate consumed.

In the presence of heme or hemoglobin in the growth medium, some LAB may change their otherwise fermentative mode of metabolism to a respiratory one, including the formation of cytochromes, the use of oxygen as terminal electron acceptor and increased ATP production, presumably by oxidative phosphorylation. This was first noted and to some extent investigated in enterococci,[31–33] but in fact also observed in other LAB such as lactococci and leuconostocs.[212] These observations were long overlooked, but the theme was revived with the complete sequencing of the *Lc. lactis* IL1403 genome[18,213] and the discovery that this strain harbored the genes for cytochrome oxidase (bd). Data have now accumulated indicating that the respiratory mode of growth of lactococci could be preferred to the "normal" fermentative mode.[37] It is also quite common among LAB to synthesize a "true" catalase in heme-containing media.[29] Apparently, many LAB have the capacity to synthesize the apoenzymes of catalase and/or cytochromes, but as mentioned previously, they are unable to synthesize porphyrins. The true extent of this will certainly be revealed in the near future as many more LAB genomes will be available.[20]

In summary, oxygen interacts with LAB metabolism in a very active way, and it has been argued that it is perhaps inaccurate to designate them mere aerotolerant anaerobes,[33,165,214] an opinion that has some support, both with regard to the actual relationship to oxygen as described above and in the phylogenetic position of these bacteria (see Sec. III.D).

However, it should be noted that LAB generally do not have the same potential to protect themselves against the toxic effect of oxygen as genuine aerobic organisms. LAB are generally devoid of catalase, although H_2O_2 may be decomposed by a pseudocatalase in some strains[29] or by a heme-catalase under certain conditions by others (see above). Since H_2O_2 is produced by NADH oxidases, accumulation may reach autoinhibitory levels depending on strain.[165] Superoxide dismutase is present in some LAB, e.g., lactococci and enterococci, and absent in others, e.g., lactobacilli. Some lactobacilli have developed a unique system for protection against superoxide. This system is based on specific accumulation of Mn^{2+} to high intracellular concentrations (30–35 mM), which have a scavenging effect on superoxide (for a review, see Ref. 215). *Lb. plantarum* is the most studied species in this regard and was recently shown to harbor genes for several transport systems for cations.[19] Some of them have been characterized to some extent.[216,217] It appears that other LAB associated with plant material (rich in Mn^{2+}), such as leuconostocs and pediococci, also possess manganese-accumulating systems.[215]

D. Other Electron Acceptors

LAB are not restricted to oxygen as an external electron acceptor. Anaerobically, several organic compounds can serve the same purpose. This is especially true for the

heterofermentative LAB. It is perhaps a fair statement to say that this group of LAB has a more obvious use for external electron acceptors, even under "normal" glucose fermentation, since this (in theory) can double the amount of ATP produced per glucose consumed. In this regard, acetyl phosphate holds a key position as intermediate in the 6-PG/PK pathway. The presence or absence of an external electron acceptor will decide whether ethanol (no ATP) or acetate (1 ATP) is formed. Lucey and Condon[205] have suggested that the ethanol branch of the 6-PG/PK pathway in no more than a "salvage route," permitting growth when an external electron acceptor is missing. The fact that some heterofermentative LAB have a defect ethanol branch (see above) and are, therefore, dependent on an external electron acceptor (at least for glucose fermentation) may support this theory.

The changes that occur in the end product formation of heterofermentative LAB in the presence of an external electron acceptor, compared to in the absence, do not have a counterpart with homofermenters. Under normal growth conditions, only minor deviations of the homolactic fermentation may occur as a result of the use of external electron acceptors. Such a deviation may be production of some acetoin as a result of a build-up of excess pyruvate. However, organic electron acceptors can play an essential role for homofermentative LAB in anaerobic metabolism of certain substrates. Examples of organic external electron acceptors that can be used by LAB are acetaldehyde, α-ketoacids, citrate, fructose, fumarate, and glycerol.[209,218–223] Further details on the metabolism of some of these will be discussed below.

1. Citrate

Citrate is not used directly as an electron acceptor, but acts as a precursor to one. The essential step is a cleavage of citrate by citrate lyase to form acetate and oxaloacetate. Different LAB use different pathways in the further metabolism of these products. Decarboxylation of oxaloacetate, yielding pyruvate, has already been mentioned in connection with the diacetyl/acetoin pathway (see Sec. IV.B). Growing cells of heterofermentative LAB dissimilating citrate in a cofermentation with carbohydrate do not form significant amounts of diacetyl or acetoin.[224,225] Rather, the excess pyruvate is reduced to lactic acid. This spares acetyl phosphate from being reduced to ethanol, and more ATP can be formed through the acetate kinase reaction, resulting in a more efficient glucose utilization and increased growth rate.[225]

The products of the citrate lyase reaction are used differently in other LAB. A pathway for succinic acid formation from oxaloacetate was proposed and proven by Chen and McFeeters.[219] This metabolism was found in the anaerobic fermentation of mannitol by *Lb. plantarum*, in which an external electron acceptor was required for growth.[219,221] This way of utilizing citrate may be more common among LAB, especially heterofermentative, than the special case of *Lb. plantarum* mannitol fermentation would suggest. It may even explain some of the confusing results with regard to carbon recoveries and fermentation balances in studies of LAB and citrate dissimilation.[224] Due to its industrial importance, most studies on this subject have been aimed at the understanding of acetoin and diacetyl formation,[224] and other fates of citrate may have been overlooked. In studies of intestinal lactobacilli, it was initially noticed that some unidentified heterofermentative strains produced succinic acid in normal, MRS-like media.[226] Later, it was shown that this was due to a utilization of citrate in a cofermentation with glucose[227] and that these strains belong to a new species of lactobacilli, *Lb. mucosae*.[228] Although enzymatic evidence is missing, the

end product formation for one of these strains, shown in Fig. 5, is consistent with the operation of a citrate lyase and the succinic acid pathway. In addition, the typical stimulation of growth rate and glucose utilization occurred, indicating increased ATP production (L. Axelsson, unpublished). A hypothetical pathway of this metabolism is depicted in Fig. 6. It was noticed that succinic acid production from citrate was fairly common among heterofermentative lactobacilli isolated from the intestine of several animals (L. Axelsson and S. Lindgren, unpublished). The property seems to be common in plant-associated heterofermentative lactobacilli as well.[229]

Citrate is also used as an electron acceptor in an anaerobic degradation of lactate, which can be performed by some strains of *Lb. plantarum*.[230] This metabolism is very slow and can only be observed after prolonged incubation. In fact, no growth is evident, but cells performing the metabolism (as evidenced by HPLC) have significantly higher ATP content than control cells (S. Lindgren and L. Axelsson, unpublished). This may point to an importance of this metabolism for survival and maintenance. The products of the cometabolism of lactate and citrate are succinic acid, acetate, formate, and CO_2, indicating the operation of both the succinic acid pathway and a pyruvate-formate lyase.[230] This metabolism has been further studied in a strain of *Lb. pentosus.* Oxygen, nitrate, and nitrite were shown to inhibit formate production, possibly acting on the pyruvate-formate lyase.[231]

The differences between LAB regarding the use of citrate may depend on the presence or absence of the enzyme oxaloacetate decarboxylase. If present, citrate utilization (the primary step being the lyase reaction) results in an increase in the pyruvate pool, which may lead to an altered end product pattern, e.g., acetoin production. If oxaloacetate decarboxylase is absent, an alternative route is the succinic acid pathway. A similar comparison was made between *Enterobacter* sp. (citrate fermenting) and *Escherichia coli* (citrate nonfermenting) when it was shown that *E. coli* could dissimilate citrate, but only in the presence of a cosubstrate, with the formation of succinic acid.[232]

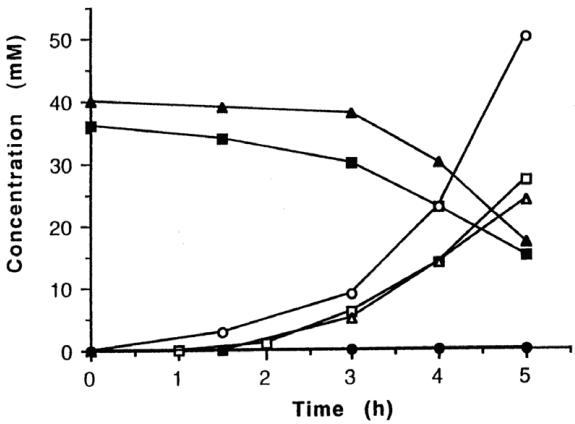

Figure 5 End product formation by *Lb. mucosae* Lbp 1031 during growth on glucose and citrate in a modified MRS medium. Samples were withdrawn from the culture and subjected to HPLC analysis. During the sampling time, OD_{600} increased from 0.2 (0 h) to 1.7 (5 h). Symbols: ■, glucose; ▲, citrate; □, lactate; ○, acetate; △, succinate; ●, ethanol.

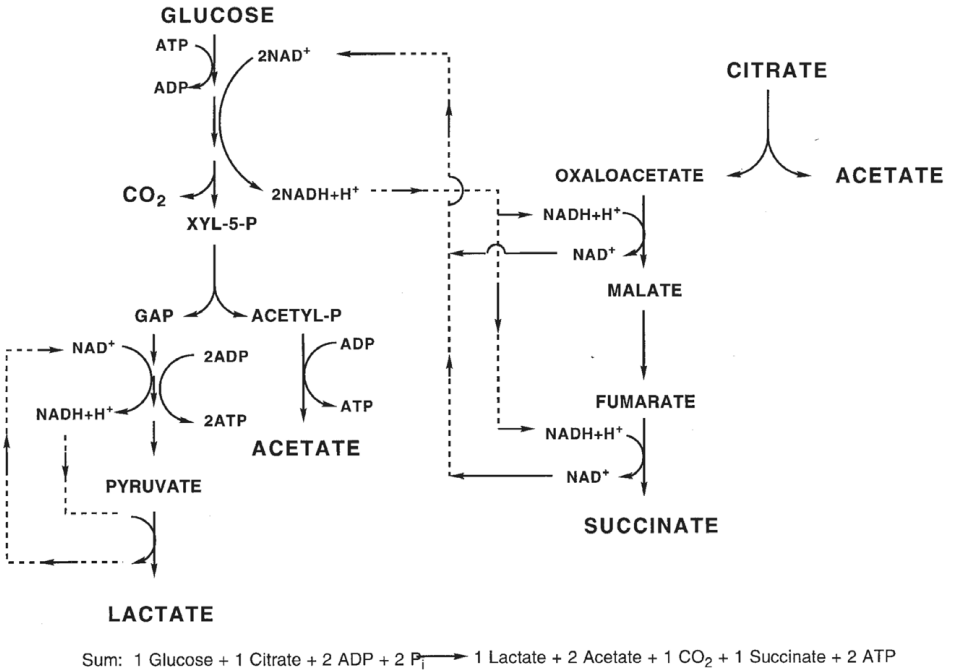

Figure 6 Proposed pathway for succinic acid production in heterofermentative lactobacilli growing on glucose and citrate.

2. Glycerol

Strains of *Lb. brevis*, *Lb. buchneri*, *Lb. collinoides*, and *Lb. reuteri*, all heterofermentative, can use glycerol as an electron acceptor in an anaerobic cofermentation with glucose. As mentioned previously, many strains of *Lb. brevis* ferments glucose poorly anaerobically. Some strains do ferment glucose, however, if glycerol is added.[218] The products of the cofermentation are lactate, acetate, CO_2 and 1,3-propanediol. Again, the NADH formed during glucose fermentation is not reoxidized by the ethanol pathway, but rather by using glycerol as electron acceptor. Glycerol is first dehydrated to 3-hydroxypropionaldehyde (3-HPA) and further reduced to 1,3-propanediol by a NAD^+ : 1,3-propanediol dehydrogenase.

Lb. reuteri ferments glucose alone with lactate, ethanol, and CO_2 as end products, but changes to more acetate/less ethanol when glycerol is added.[223] The addition of glycerol also stimulates growth rate and Y_{glc} is increased. The same pathway for glycerol reduction to 1,3-propanediol as for *Lb. brevis* was shown to be functional in *Lb. reuteri*.[223,233]

Some differences in response to glycerol between these species can be noted. Resting cells of *Lb. brevis* metabolizing glycerol accumulate 1,2-propanediol,[218] whereas *Lb. reuteri* under these conditions accumulates and excretes the intermediate 3-HPA. This compound is a potent antimicrobial substance, initially termed reuterin.[234–236] The enzymes of the glycerol pathway seem to be under some kind of regulation in *Lb. brevis*, since cells grown on pentoses do not contain any glycerol dehydratase activity.[237]

This is logical, considering that pentose fermentation does not generate excess NADH. On the contrary, *Lb. reuteri* has about the same levels of this enzyme whether grown on pentose or glucose, despite the fact that the glycerol pathway is not used when the cells ferment pentoses (M. Fiuzat, L. Axelsson, and W. Dobrogosz, unpublished).

In the presence of glycerol, strains of *Lb. brevis* and *Lb. collinoides* also ferment the initial lactate formed from glucose with acetate, ethanol, CO_2, and 1,3-propanediol as end products.[238,239]

The growth response of *Lb. reuteri* with the addition of glycerol (increased growth rate and Y_{glc}), is very similar to that observed when oxygen is used as electron acceptor by *Ln. mesenteroides*.[205] The increase in Y_{glc} is easy to understand, since more ATP per glucose can be formed through the acetate kinase reaction. But why is the growth rate increased? No clear-cut answer can be given, but Lucey and Condon[205] suggested that ATP formation is the rate-limiting step in these bacteria.

3. Fructose

It was early noted that fructose fermentation by heterofermentative LAB resulted in mannitol formation.[240] This metabolism represents an interesting example of a case where the same compound acts as both the growth substrate and electron acceptor.[209] Fructose is fermented by the normal 6-PG/PK pathway, but some of the sugar is reduced to mannitol by a NAD^+ : mannitol dehydrogenase.[209] Similar to the use of other external electron acceptors, this enables the cells to produce ATP through the acetate kinase reaction. Assuming no ethanol formation, the overall equation for fructose fermentation would be:

$$3\ \text{fructose} + 2\ \text{ADP} + 2\ P_i \longrightarrow 1\ \text{lactate} + 1\ \text{acetate} + 1\ CO_2 + 2\ \text{mannitol} + 2\ \text{ATP} \quad (1)$$

As can be seen, this is less efficient than glucose fermentation in terms of ATP formed per sugar consumed. However, for some heterofermentative lactobacilli, the growth rate is higher on fructose than glucose (L. Axelsson, unpublished). This indicates that under conditions of substrate excess, a priority is given to growth rate rather than efficiency of substrate utilization. The reduction of some of the fructose to mannitol could play a role, since the cells may be able to form more ATP per time unit. The metabolism may be of great importance in natural plant fermentations, where glucose, fructose, and sucrose are the main sugars.[241] Heterofermentative LAB can use glucose as energy source and fructose as electron acceptor, thus obtaining optimal growth rate. A maltose-fructose cofermentation has also been suggested to improve sourdough fermentation.[242] Renewed interest in this metabolism of LAB has recently occurred as a result of the suggestion to use mannitol as a "nutraceutical," i.e., an active ingredient in functional foods.[243,244]

E. The Malo-Lactic Fermentation

The metabolism of L-malic acid (the D form is not attacked) by LAB has been extensively studied. This has been due to its technological significance, mainly in wine manufacture, but also because it has presented some interesting physiological problems with regard to metabolism and bioenergetics.

Few LAB, e.g., *E. faecalis* and *Lb. casei*, can use malate as the sole energy source. The NAD^+-dependent malic enzyme catalyzes the decarboxylation of malate to pyruvate

and CO_2[182] as follows:

$$\text{Malate} + \text{NAD}^+ \longrightarrow \text{pyruvate} + CO_2 + \text{NADH} \quad (2)$$

Pyruvate is converted to acetate, ethanol, and CO_2 with ATP generation presumably through the acetate kinase reaction.

The more interesting, and more common in LAB, malate metabolism is the conversion of malate to lactate and CO_2, which proceeds in a cofermentation with a fermentable carbohydrate. This fermentation, often referred to as malo-lactic fermentation (MLF), can be performed by many LAB. MLF is significant in the fermentation of vegetables and fruits, where malate is present in fairly high concentrations.[107] The conversion of malate to lactate in the late stages of wine-making is well known, where it may be desirable or undesirable, depending on the wine variety.[111]

The pathway for malate dissimilation was first believed to be a sequence of reactions, the first being identical to Eq. (2), resulting in pyruvate as a free intermediate. Pyruvate would subsequently be reduced to lactic acid by the LDH. An indication of another mechanism was that MLF-leuconostocs produced exclusively L-lactic acid from malate.[111] Pyruvate could thus not be an intermediate, since leuconostocs only possess a D-LDH (see Sec. III.B). Subsequently, purification to near homogeneity of the enzyme catalyzing the complete reaction was achieved:[111]

$$\text{Malate} \xrightarrow{\text{NAD}^+} \text{lactate} + CO_2 \quad (3)$$

To distinguish the enzyme from the malic enzyme and malate dehydrogenase, it was given the trivial name malo-lactic enzyme [the proper name being L-malate : NAD^+ carboxylyase[245]]. Curiously, although NAD^+ was required for enzymatic activity, no NADH was detected in early studies of the enzyme. This would indicate that hydrogen exchange reactions occurred within the enzyme and that oxaloacetate and/or pyruvate may be intermediates, but bound to the enzyme.[164]

LAB performing the MLF in cofermentation with a carbohydrate generally benefit from this in a way that resembles the use of external electron acceptors (see Secs. IV.C and IV.D), i.e., increased growth rate and a higher Y_{glc} compared to growth on solely glucose.[246] This is difficult to explain, since apparently no potential electron acceptors such as oxaloacetate or pyruvate are produced by the reaction. The stimulatory effect was first attributed to the deacidification of the external medium,[111] which is a consequence of the reaction since malic acid has a lower pK_a than lactic acid.[247] This may be of importance, but cannot fully account for the stimulatory effect. Later studies have shown that the reaction is not stoichiometrically complete and that small, but significant amounts of pyruvate and NADH are released.[245] The reaction can thus provide additional electron acceptors. Measurements of internal ATP concentrations in cells performing the MLF have also indicated that the reaction confer benefits in the form of energy.[248] These authors suggested that this effect is indirect in that MLF relieves the cells from some of the energy requirements, in particular that required for generating and maintaining a proton gradient. This could be achieved by the "energy recycling model" of lactate efflux, but was subsequently shown to be mainly a consequence of so-called electrogenic precursor/product exchange (for further discussion on energetics, see Sec. V.A).

F. Nitrogen Metabolism: The Proteolytic System

It is a general belief that LAB have a very limited capacity to synthesize amino acids using inorganic nitrogen sources. They are, therefore, dependent on preformed amino acids being present in the growth medium as a nitrogen source. It should be noted, however, that the requirement for amino acids differs among the species and strain variations exist within species. Some strains of *Lc. lactis* subsp. *lactis* are in fact prototrophic for most amino acids, while *Lc. lactis* subsp. *cremoris* and *Lb. helveticus* strains require 13–15 amino acids.[249] The requirement for a particular amino acid may be the result of mutations in the genes for amino acid biosynthesis and/or the downregulation of these genes or the involved enzymes.[249,250] Growth on chemically defined minimal media is generally slow, and it is clear that LAB have adapted to rich environments by developing systems to efficiently exploit the nitrogen sources present there. One of the most extensively studied systems in this regard is the proteolytic system of dairy LAB, in particular that of *Lc. lactis.* The reason is of course the technological significance in milk fermentation, as it has been shown that a proteolytic system is necessary for appreciable and rapid growth in milk. There are several reviews covering this topic in more depth (e.g., Refs. 251,252). Below follows a short summary.

All dairy lactococci used for acidification of milk (e.g., in cheese manufacture) have proteolytic activity. An extracellular, membrane-anchored serine proteinase (PrtP) was identified as being essential for this activity. Mutants defective in the gene of this protein grow to only very low densities in milk. PrtP exists in at least two variants in lactococci with somewhat different specificities in the degradation of milk casein. Several peptidases with different specificities have been identified in lactococci, but to date all well-characterized peptidases have been found to be intracellular. Earlier studies noticed a "missing link" in the proteolytic pathway, since the peptides produced by the activity of PrtP were too large to be directly transported by the transport systems identified. These are amino acid transport systems,[22] two di- and tripeptide transport systems,[253] and an oligopeptide transport system (Opp) accepting 4- to 8-amino acid residue peptides.[254] Using mutants, one study also suggested that a broad-specificity di-/tripeptide transport system was essential for casein utilization.[255] The problem was then as follows: since the products of casein degradation appear to cross the membrane as di- or tripeptides and PrtP only produces larger oligopeptides, there should be an extracellular peptidase involved in the pathway. As mentioned, such a peptidase has never been convincingly identified. Refined tools for analyzing the products of casein degradation by PrtP, the advanced knowledge of the genetics of the system, methodologies for constructing well-defined mutants (e.g., inactivation by chromosomal integration), and thorough studies of the Opp system subsequently solved this problem. First, casein was shown to be degraded by PrtP to a much larger extent than previously thought, with oligopeptides smaller than 9 residues being a large fraction.[256] Second, using well-defined mutants, it was clearly shown that the Opp system was essential for growth on casein, while the di-/tripeptide transport system was not.[257] Third, by analyzing the peptide fraction in milk during growth, it was calculated that oligopeptides small enough to be accepted by the oligotransport system represent 98% of the nitrogen source for growth of lactococci in milk.[258] Fourth, the Opp system was shown to accept oligopeptides up to 18 residues, further strengthening the role of this transport system.[259] A model for the complete proteolytic pathway can now be constructed and is schematically shown in Fig. 7. The gene for PrtP, performing

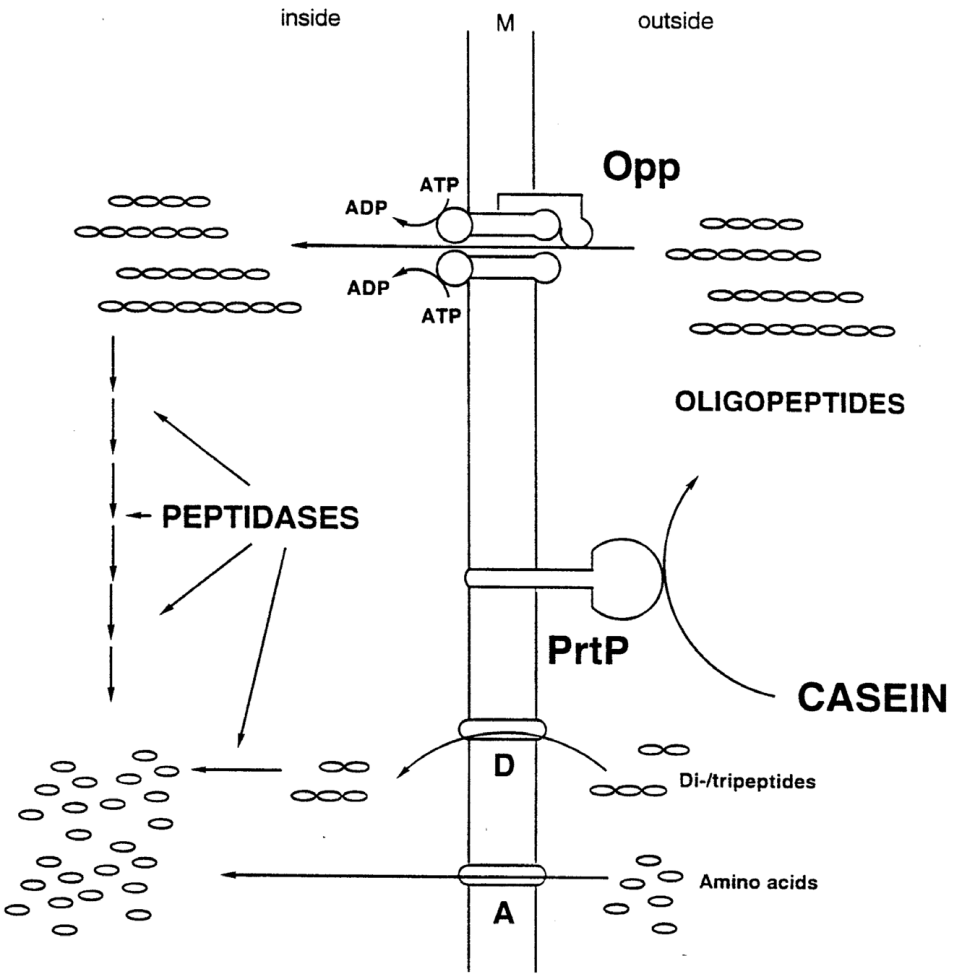

Figure 7 Model of the proteolytic pathway in *Lc. lactis.* Included is transport of di- and tripeptides and free amino acids, but note that these contribute very little to the total growth of lactococci in milk (see text). PrtP, membrane-anchored proteinase; Opp, oligopeptide transport system; D, di-/tripeptide transport system(s); A; amino acid transport system(s); M, cytoplasmic membrane.

the prime step in the pathway, has been shown to be regulated by specific dipeptides,[260] presumably produced by the action of intracellular peptidases from transported oligopeptides in the in vivo situation. The extent to which this knowledge of the lactococcal proteolytic system can be transferred to other LAB is not known at present, since detailed studies in other species are scarce. As far as they do exist, they seem to indicate that the proteolytic systems are indeed similar to lactococci.[251] Except for the primary enzyme in the pathway, the extracellular proteinase, the genome of *Lb. plantarum* contains all genes necessary for protein degradation, i.e., genes encoding for the Opp system and and array of peptidases of different specificity.[19] Other lactobacilli do possess a PrtP protein very similar to the lactococcal counterpart.[261]

V. ENERGY TRANSDUCTION AND SOLUTE TRANSPORT

Members of LAB have been extensively studied with regard to mechanisms of transport and energetics, in particular species of the genera *Enterococcus*, *Lactococcus*, and *Streptococcus*. Model systems, which use these bacteria, are easy to control and manipulate. Aerotolerance, an efficient fermentative metabolism with no oxidative phosphorylation, and a cell wall without outer membrane are valuable properties in this regard. The results obtained with these model systems have been important for the understanding of living cells in general.[23] Sugar transport is of course connected to the carbohydrate metabolism described in previous sections, but the discussion on the subject has been placed here because transport systems in general are tightly coupled to the bioenergetics of the cells.

There are excellent reviews covering this vast field (e.g., Refs. [21–26,171,262, 263]), and the presentation here can only be a short summary.

A. Bioenergetics of LAB

1. ATP, the Proton Motive Force, and Internal pH

The metabolism of LAB is aimed at the generation of ATP, the universal energy carrier in all living cells. ATP, or high-energy compounds interconvertible with ATP, is needed for the thermodynamically unfavorable "reaction" of building cells. The most important energy-requiring events in cells are the synthesis of macromolecules and the transport of essential solutes against a concentration gradient. The so-called chemiosmotic theory in bioenergetics[264,265] is now generally accepted. Cellular metabolism leads to an electrochemical proton gradient across the cytoplasmic membrane. The most commonly known system for creating a proton gradient is the membrane-linked electron transport chain, present in respiring organisms. The flow of electrons through the system via different carriers in effect pumps protons out of the cell. The proton gradient across the cytoplasmic membrane is composed of two components, an electrical potential ($\Delta\Psi$), inside negative, and a pH gradient (ΔpH), inside alkaline. $\Delta\Psi$ and ΔpH exerts an inwardly directed force termed the proton-motive force (PMF). In organisms with an electron transport chain, this force is large enough to be converted into chemical energy, i.e., ATP. This is accomplished by a membrane-located enzyme, the H^+-translocating ATPase, or in this function also known as the ATP synthase. The energy of the reversal flow of protons "through" the enzyme, into the cell, is used to form ATP from ADP and phosphate.

LAB do not possess an electron transport chain (at least not in the absence of preformed heme) and are hence not able to form ATP in this way. Instead, ATP is generated by substrate-level phosphorylation, which is characteristic for all fermentative organisms. LAB do, however, possess an enzyme very similar to the ATP synthase, but the major role of this enzyme is the reverse reaction, i.e., the hydrolysis of ATP with concomitant pumping of protons out of the cells.[22,23] LAB (and fermentative bacteria in general) thus establish a PMF, which can drive energy-consuming reactions such as the uphill transport of metabolites and ions.

The difference in function between the H^+-ATPase of LAB and the ATP synthase of respiring organisms is merely a reflection of the different modes of metabolism. The H^+-ATPase of *Lc. lactis* is capable of acting as an ATP synthase under certain conditions.[24,37,266] It was also quite early established that the H^+-ATPases of *E. faecalis,*

Lc. lactis subsp. *cremoris*, and *Lb. casei* have the same basic structure as the ATP synthase from mitochondria, chloroplasts, and respiring or photosynthesizing eubacteria.[267–269]

It is well known that bacteria attempt to maintain the cytoplasmic pH (pH_i) at a certain level or interval.[270] LAB are to a certain degree exceptions to this, since they tolerate lower and a wider range of internal pHs. There are, however, differences within the LAB group. In general, lactobacilli are significantly more tolerant to low pH_i than enterococci, leuconostocs, and streptococci.[21,271] It is logical that the enzymes and the general machinery of the cells have threshold levels below (and above) which they cannot function. It appears that some lactobacilli have developed a very "relaxed" system, which works even at a pH_i of 4.2–4.4. In contrast, enterococci, lactococci, and streptococci rarely tolerate a pH_i lower than 5.0.[21,22] Since the external pH (pH_o) falls well below these values, due to massive acid production, a mechanism must exist to maintain pH_i above the threshold levels. Studies of *E. faecalis* suggest that the H^+-ATPase plays a crucial role and that this role is the main function of the enzyme.[272] The extrusion of protons by the H^+-ATPase and the electrogenic uptake of K^+ maintain the cytoplasm more alkaline than the outside medium. Both the activity and the synthesis of the H^+-ATPase are regulated by pH_i. The mechanism of pH homeostasis in lactococci seems to be very similar to the *E. faecalis* system.[22]

2. "Energy Recycling" and Electrogenic Precursor/Product Exchange

The maintainance of a pH_i above the threshold level and the generation of a PMF require the use of a substantial part of the ATP generated by substrate-level phosphorylation. This makes less ATP available for biosynthetic purposes. Any other means of generating or maintaining the PMF than the H^+-ATPase would save energy. Some LAB have developed such systems, which to some degree compensate for the drain of ATP.

A general scheme for PMF-driven transport is depicted in Fig. 8A. The inwardly directed gradient of protons is the driving force for the influx of a solute X, which enters the cell together with a proton (proton symport). The question is whether this process, in its principles, is reversible, as shown in Fig. 8B? Here, the outwardly directed gradient of solute Y drives the efflux in symport with a proton, thus creating a PMF. The answer to the question is yes, under certain conditions. Based on theoretical calculations, where solute Y (Fig. 8B) was represented by a fermentation end product (such as lactate), a model was proposed as to how energy could be conserved by end-product efflux, the so-called "energy recycling model".[273] If the efflux is electrogenic, i.e., a net charge leaves the cell together with the end product, a PMF is generated. In the case of lactate, more than one proton (on average) per lactate molecule has to be exported to obtain an electrogenic efflux. Experimental results with *Lc. lactis* subsp. *cremoris* have supported the model.[274,275] A carrier-mediated electrogenic lactate efflux was shown to occur at pH_o above 6.3 and low external lactate concentrations (<10 mM). At lower pH_o and higher external lactate concentrations, the lactate efflux was electroneutral and subsequently not contributing to the generation of a PMF. In practice, this energy-saving process is only operational at the initial stage of growth in a batch culture. In an ecological context, the significance of the process may be substantial, since an initial high growth rate (which presumably would be the result of more ATP available for biosynthesis) could be advantageous in competition with other microorganisms. A dramatic effect was observed when *Lc. lactis* subsp. *cremoris* was grown in coculture with a lactate-consuming organism, *Pseudomonas stutzeri.* The conditions for maximium advantage of lactate efflux

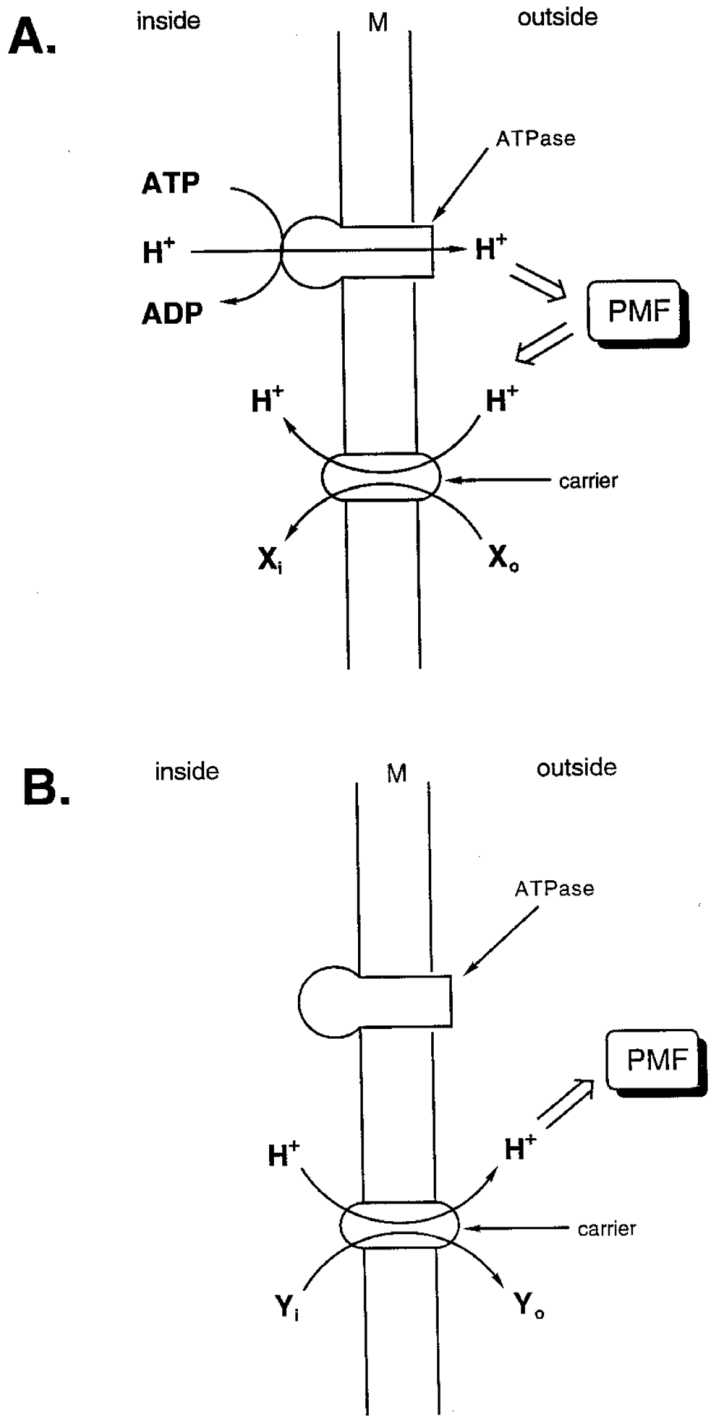

Figure 8 Schematic representation of proton-motive force (PMF) formation by a H^+-ATPase and PMF-driven transport (A), and electrogenic end product efflux (B). M denotes the cytoplasmic membrane.

(low external lactate concentration and high pH_o) were thus maintained throughout growth and a 60–70% increase of the molar growth yield for lactose was obtained.[276]

The energy recycling model has been shown to be applicable also for acetate efflux in *Lb. plantarum*.[277] This species responds to a shift from anaerobic to aerobic conditions by producing some acetate at the expense of lactate and by increasing the molar growth yield for glucose.[278] An electrogenic acetate efflux was demonstrated, and this, together with additional ATP formation via the acetate kinase reaction, was suggested to contribute to the energy economy of the cells.

The system of energy recycling by lactate efflux is not general for all LAB. A carrier-mediated, electroneutral export of lactate was shown for *Lb. helveticus*.[279] This species is able to produce very large amounts of lactic acid (>200 mM) and the pH_o drops to levels well below 4.0. The massive lactic acid production and acid tolerance of *Lb. helveticus* is very different from lactococci, and it is perhaps not surprising that the lactate export system differs. It is not known if the system of *Lb. helveticus* is typical for the more acid-tolerant LAB.

Recently, another mechanism for creating a PMF has been identified in LAB. These findings stem from the attempts to explain the energy benefits of the malo-lactic fermentation (see Sec. IV.E). It has been shown that MLF in *Lb. plantarum* and *Lc. lactis* functions as an indirect proton pump.[280,281] The precursor (malate) is exchanged for a product (lactate) with higher charge, making the overall reaction electrogenic. The key is the compartmentalization of the pathway, i.e., the decarboxylation occurring inside the cell and consuming one proton.[24] The PMF formed by the MLF alone is high enough to drive ATP synthesis by the H^+-ATPase, but may under normal conditions, i.e., cofermentation of sugar malate, be a mechanism to conserve rather than directly generate energy.[24] A generalization of these so-called electrogenic precursor/product exchange reactions is shown in Fig. 9. The exchange reaction may be direct, mediated by one exchange protein (Fig. 9A), or indirect, via one precursor uptake protein and one product exit protein (Fig. 9B). The benefits of citrate metabolism in *Lc. lactis* can be explained by the same mechanism, where essentially a di- or trivalent anion (citrate) is exchanged for monovalent (acetate) and neutral (acetoin) products concomitant with decarboxylation.[282] Certain lactobacilli can produce biogenic amines such as histamine and tyramine by decarboxylation of the amino acids histidine and tyrosine, respectively. This leads to the generation of a PMF, presumably by the same general mechanism.[24]

B. Transport of Solutes

LAB are generally very fastidious and require amino acids, nucleotides or nucleotide precursors, and vitamins in addition to an energy source, generally a carbohydrate. A prerequisite for rapid growth is efficient transport systems for the uptake of essential nutrients. The transport of solutes is highly connected to the bioenergetics of the cells.

LAB use different types of transport systems, which can be broadly divided into three categories based on differences in the form of energy used in the translocation process:[26] (a) primary transport (chemical energy, e.g., ATP-driven), (b) secondary transport (chemiosmotic, e.g., PMF-driven), and (c) group translocation (chemical modification concomitant with transport), i.e., phosphoenolpyruvate : sugar phosphotransferase system (sugar PTS).

It should be emphasized that the most extensive research on transport systems among LAB has been done with *Lc. lactis*. For this species, a nearly complete picture of the transport of sugars, amino acids, peptides, and ions has emerged.[22,24] Much of the discussion below is based on these results.

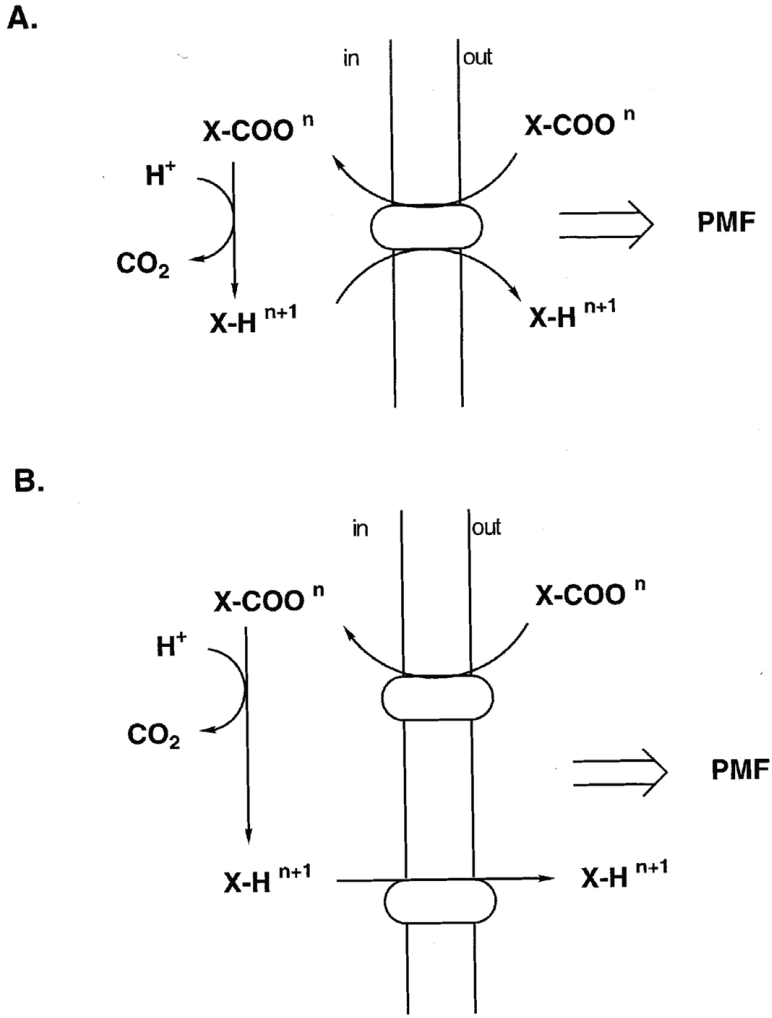

Figure 9 Schematic representation of electrogenic precursor/product exchange with intracellular decarboxylation. The precursor (X-COO) with a certain charge (n) is transported into the cell and decarboxylated to yield the product (X-H) with higher charge ($n + 1$). The product can be exported via a precursor/product antiport (A) or by a separate carrier (B). The reaction contributes to the formation of the PMF.

1. Primary Transport (Phosphate Bond–Linked Transport, ATP-Driven)

The most common class of primary transporters in LAB are members of the ABC (ATP-binding cassette)-transporter superfamily.[283] They are represented by systems for accumulating substrates and compatible solutes, such as the glutamate/glutamine transporter, the oligopeptide (Opp) transport system, and the OpuA (*Lc. lactis*/*Lb. plantarum*) or QacT (*Lb. plantarum*) transporters for defense against osmotic shock, but also by systems for excreting unwanted compounds (e.g., drugs) such as LmrA of *Lc. lactis.*

Glutamate and glutamine transport in lactococci is mediated by a system dependent on the production of metabolic energy by either glycolysis or the ADI pathway.[284,285] The activity of the transport system decreases if internal ATP levels are lowered. A single transport system for glutamate and glutamine has been identified.[285] The system has an absolute preference for the undissociated form of glutamate (glutamic acid), which leads to growth limitations at alkaline pH values.[286] Since glutamine transport is independent of pH, the cells can be relieved of this effect if higher concentrations of glutamine are included in the medium as a source of glutamate.

The oligopeptide (Opp) transport system, crucial in the proteolytic pathway (see Sec. IV.F), is ATP driven and unaffected by the magnitude of the PMF. The system has been characterized both genetically and biochemically.[254] It is composed of five proteins, two of which (OppD and OppF) show the typical ATP-binding domains of the ABC-transporter superfamily. The physiological characteristics of the glutamate/glutamine and the oligopeptide transport systems are similar, and a multiprotein complex has been predicted for the former.[24]

Microbial cells respond to osmotic up-shifts by accumulating certain compounds intracellularly to very high concentrations. These compounds do not affect the macromolecules of the cells and may in fact stabilize the structures of some enzymes. LAB use an ABC-transporter, OpuA or equivalent, for accumulating glycine betaine, which appears to be the preferred compatible solute. This feature has been mostly studied in *Lc. lactis* and *Lb. plantarum.* For a review on the subject, see Ref. 26.

2. Secondary Transport

PMF-Driven Symport. The PMF-driven transport systems are perhaps the most common and general among LAB for the transport of nutrients into the cell (as in most bacteria). The principles of this transport system have already been mentioned and are schematically drawn in Fig. 8A. A specific, membrane-associated protein (carrier, permease) translocates the solute across the membrane in symport with one proton. Presumably, many sugars are transported in this way (see also Sec. IV.A), although the actual mechanisms, specificities, etc. have not been studied to any large extent, with the exception of lactose transport. PMF-driven lactose transport has been shown in lactococci, lactobacilli, and *S. thermophilus*.[22,24,98,263,287,288] The mechanism has been studied in some detail in *S. thermophilus.* As mentioned, this species only ferments the glucose moiety of lactose, while galactose is excreted. Despite this being an exchange reaction, with energetic benefits, the actual carrier, LacS, is not a strict antiporter, as was suggested,[289] but can also transport lactose in symport with a proton with PMF as the driving force. Efflux of galactose is a result of internal accumulation (due to a defective induction of galactokinase) and the fact that the carrier also has affinity for this substrate.[24] In vivo, the exchange reaction seems to be the most relevant since it is faster than the symport reaction.[290] In certain lactococci the permease-mediated transport of lactose coexists with a lactose PTS system.[22] Similarly, it has been shown that glucose can be transported either by a PMF-driven system or a glucose PTS in a strain of *T. halophilus*.[291] The reason for having two separate transport systems for the same solute is not fully understood, but differences in affinity may allow the cells to use either system at different substrate concentrations.[22]

In lactococci, most amino acids are also transported by PMF-driven symport systems.[22] Stucturally similar amino acids, e.g., leucine, isoleucine, and valine, may

share the same carrier protein. Differences between carriers with regard to affinity and pH regulation has been shown.[22]

One of the di- and tripeptide transport systems has also been shown to be PMF driven, with a broad-specificity carrier.[255] For the amino acid proline, peptides seems to be the preferred source. This has been attributed to an efficient transport system for proline-containing di- or tripeptides (in the form of Pro-X or Pro-X-X), while an active transport system for the free amino acid is missing (i.e., a dependence on passive diffusion).

Precursor: Product Antiport. Many LAB can derive energy through substrate-level phosphorylation by the metabolism of arginine.[292–294] The pathway of this metabolism, the arginine deiminase (ADI) pathway, is shown in Fig. 10. For unknown reasons, most arginine-metabolizing LAB cannot use arginine as sole energy source,[22,113] but catabolize it simultaneously with a fermentable carbohydrate. Ornithine, one of the end products of the metabolism, is excreted into the medium.[295] It has been established, at least for lactococci, that the driving force for arginine uptake and ornithine excretion is the concentration gradients of these compounds[22] with no involvement of PMF. The stoichiometry for the arginine : ornithine exchange is strictly 1 : 1, and the exchange has been shown to be mediated by a single, membrane-associated protein, the arginine/ornithine antiporter.[296] The enzymes of the ADI pathway, except carbamate kinase (which presumably has other, anabolic roles), as well as the antiporter itself is repressed by glucose and induced by arginine.[22,296] The arginine metabolism leaves the cells without any net accumulation of amino acid, and the question arises as to how the biosynthetic need for arginine is supplied. The activity of the PMF-dependent lysine carrier is probably the answer to this question since (a) it has some affinity for ornithine, which thus might be recaptured, and (b) the antiporter can catalyze heterologous exchange of arginine for lysine in addition to exchange of arginine for ornithine.[24]

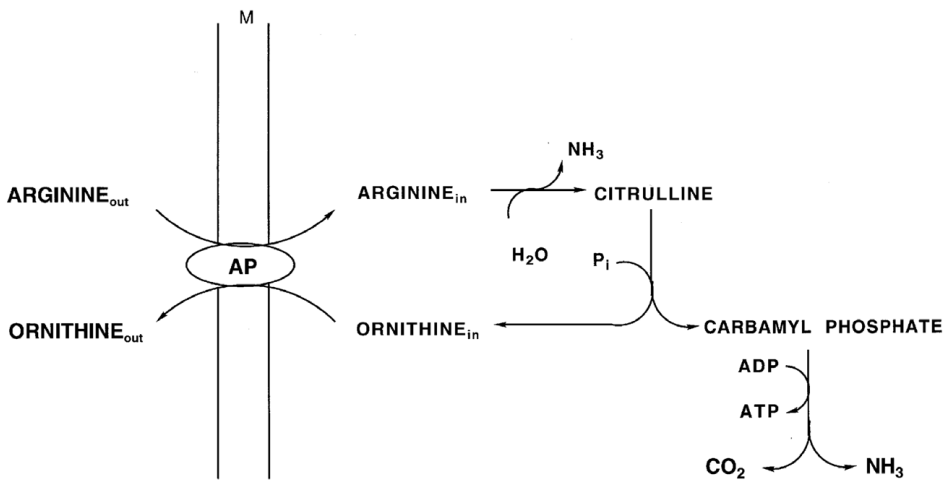

Figure 10 The arginine deiminase pathway with the arginine/ornithine antiporter (AP). M denotes the cytoplasmic membrane.

3. Group Translocation: The Phosphoenol pyruvate:Sugar Phosphotransferase System

The PTS is a complex enzyme machinery, whose main function is to translocate a sugar across a membrane with simultaneous phosphorylation (for a comprehensive review, see Ref. 297). Since there are two separate molecular species outside (sugar) and inside (sugar phosphate) the membrane, the translocation does not involve any concentration gradients. The energy for the process is provided by the high-energy phosphate bond of phosphoenolpyruvate (PEP). The energy of the phosphoryl group is transferred in a series of reactions, via PTS-specific proteins, to a membrane-located enzyme, which mediates transport and phosphorylation of the sugar.

The components and reactions of the system are depicted in Fig. 11. The first two proteins in the series, Enzyme I (EI) and heat-stable protein (HPr), are sugar nonspecific (can be shared by several PTS), whereas Enzyme IIBC (EIIBC) and Enzyme IIA (EIIA) are sugar specific (denoted by a superscript in Fig. 11). Note that in older literature, EIIBC and EIIA were denoted EIII and EII, respectively. The sugar-specific components may also exist as a fusion protein, designated EIIABC, or as separate proteins IIA, IIB, and IIC. The suffixes represent different domains containing active sites involved in the reactions. EIIBC is a membrane-located enzyme that acts in concert with the phosphorylated EIIA to mediate recognition, translocation, and phosphorylation. EIIBC and EIIA are sugar specific, but the specificity may not be absolute. Thus, the glucose PTS in most LAB also recognizes mannose and is often designated man-PTS to distinguish it from the glc-PTS, first described in *E. coli*, which has a different substrate specificity.[297]

The distribution of PTS in bacteria has been discussed by several authors.[263,297–299] There has been a general agreement that the presence of PTS is highly correlated to the ability to ferment substrates through the Embden-Meyerhof-Parnas pathway, i.e., glycolysis. It has been argued that the production of 2 mol of PEP per hexose consumed (as in glycolysis) is needed for a functional metabolism, including a PTS.[299] LAB have

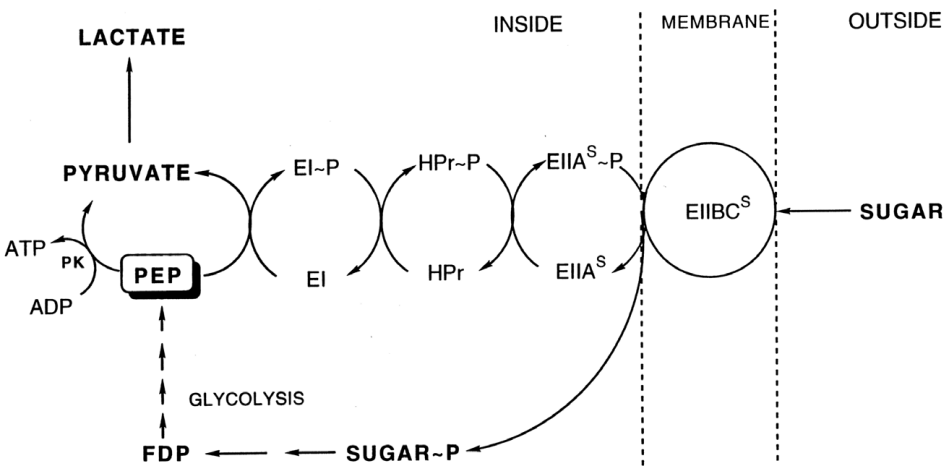

Figure 11 Sugar transport mediated by the phosphoenolpyruvate : sugar phosphotransferase system (PTS) and relation to glycolysis. PK, pyruvate kinase. See text for details.

provided some support for this in that sugar PTS were not found at first in heterofermentative LAB.[263,299] However, PTSs for gluconate and pentitols have been described in LAB (see Sec. IV.A). These compounds are fermented by the 6-PG/PK pathway and hence do not result in two PEP per substrate. In addition, reports clearly show that heterofermentative LAB also can have sugar PTS activity, although it may be uncommon.[183,300] As a generalization, the sugar PTS is tightly coupled to glycolysis in most bacteria. This is schematically shown in Fig. 11. The system actually constitutes a cycle, in which PEP holds a key position.[262] The transport of the sugar is directly coupled to the subsequent metabolism, which in turn provides the PEP needed for a new cycle to begin.

As depicted in Fig. 11, PEP has two alternative fates. It can either donate the phosphoryl group to EI and initiate the PTS cycle, or it is used by pyruvate kinase to form ATP. Pyruvate kinase is subject to regulation, where FDP acts as activator and P_i as inhibitor.[262] Under optimum glycolyzing conditions, when the FDP level is high and the P_i level low, pyruvate kinase is most active and the concentration of PEP is low.[262] A decrease in the glycolytic rate as a result of limiting concentration of sugar will result in a decrease in FDP levels and an increase in P_i.[301] Consequently, pyruvate kinase activity decreases and the concentration of PEP increases. During complete starvation, when both pyruvate kinase and the PTS are inoperative, the cells contain high concentrations of PEP and the preceding intermediates in the glycolytic pathway, 3-phosphoglycerate and 2-phosphoglycerate.[262] This pool of metabolites, designated the PEP potential, enables the cells to quickly resume transport and glycolysis once a sugar becomes available. The PEP potential may also be important in providing maintenance energy during starvation by a residual activity of pyruvate kinase.[262]

VI. REGULATION OF CARBON METABOLISM

I found it appropriate to place this short section on the regulation of carbon metabolism in LAB not only after the general sections on metabolism, but also after the section on energy transduction and transport. Carbon metabolism regulation has been shown to be an interplay between several components that have roles in more than one context, thus connecting transport of solutes, transcriptional control, and catabolism. LAB have been valuable as model systems for basic research on how carbon metabolism is regulated in bacteria. One of the foundations for this was laid with the basic knowledge obtained by research on PTS-mediated transport (for reviews, see Refs.[171,242,263]). Regulation of carbon metabolism is complex and features intriguing regulatory circuits.[263,302] Within the scope of this chapter, only a few of those features can be mentioned.

To avoid unnecessary enzyme synthesis and to achieve maximum profit from a substrate mixture, bacteria have developed mechanisms to monitor nutritional and energy status and to respond to these conditions. "Coarse control" is achieved by a substrate induction, i.e., most carbon sources act as inducers for transcription of the genes encoding transport and/or catabolic systems for that particular substrate. However, facing a substrate mixture, this is not sufficient as a regulatory response since all the (potential) inducers are present. By global transcriptional control, bacteria utilize carbon sources in a hierarchial manner and repress the genes necessary for catabolizing the less preferred substrate. This is achieved by a mechanism known as carbon catabolite repression (CCR). CCR involves both direct transcriptional control via trans-acting repressor proteins

and mechanisms that keep the inducer concentration in the cytoplasm low, thereby indirectly affecting gene expression. Global control of carbon metabolism has been extensively studied both in enteric bacteria (e.g., *Escherichia coli*) and in the low G + C gram-positive bacteria, mainly *Bacillus subtilis*, but also, as mentioned, in LAB. The mechanisms of CCR and carbon control in these two major groups of bacteria are partly different (for a review, see Ref. 303), but the phenotypes are similar in both systems, and components of the PTS system play a pivotal role.

Central to CCR in gram-positive bacteria is catabolite control protein A (CcpA). CcpA is a trans-acting repressor of several catabolic operons involved in the utilization of carbon sources other than glucose.[304,305] However, CcpA can also act as an activator of transcription in some instances. CcpA exerts its function through binding to the *cis*-acting sequence called catabolite-responsive element (*cre*), usually present in or in the vicinity of the promoter region of the pertinent gene/operon. It has been firmly established that the link between the energy status in the cells and CCR in gram-positive bacteria, which in gram-negative bacteria is performed mainly by Enzyme IIA^{Glc} of the PTS system, is the PTS protein HPr together with a HPr kinase/phosphatase (HPrK/P).[26,303] The phosphorylation of HPr by EI ~ P in the PTS cycle depicted in Fig. 11 takes place at the histidine-15 residue, resulting in HPr(His ~ P). However, HPr can also be phosphorylated at serine-46 by HPrK/P, resulting in HPr(Ser ~ P). High PTS activity and high catabolic rate will increase the pool of FDP which stimulates HPrK/P kinase activity in some LAB. In others, high ATP levels, also indicative of high catabolic rate, stimulate the kinase. The phosphorylation of HPr to HPr(Ser ~ P) will break the PTS cycle and ultimately reduce sugar uptake and catabolism. Lower levels of glycolytic intermediates will turn off the kinase activity of HPrK/P and instead activate its phophatase activity, which frees HPr for its PTS function again. Thus, the phosphorylation state of HPr reflects the metabolic state of the cell, and this is achieved not only for PTS sugars. The role of HPr is emphasized by the fact that HPr(Ser ~ P) is the activator of the CcpA protein. A complex between HPr(Ser ~ P) and CcpA is first formed and the complex binds specifically to the *cre* site. Interestingly, CcpA acts as activator of glycolytic enzymes, such as the *las* operon in *Lc. lactis*.[306] This study also showed that a CcpA mutant performed a mixed acid fermentation. The phosphorylation status of HPr and its effect on CcpA could thus be a link between the levels of glycolytic intermediates and mode of fermentation, instead of a direct allosteric effect on nLDH, as first suggested (see Sec. IV.B).

As mentioned, CCR also encompass ways of keeping intracellular inducer concentrations low. Two mechanisms can be distinguished: *inducer exclusion* and *inducer expulsion*, i.e., to avoid uptake of one sugar when a preferential substrate is present and to excrete already accumulated inducer when a preferential substrate becomes available, respectively. The inducer exclusion effect was shown quite early in lactococci using the galactoside analogue thiomethyl-β-D-galactopyranoside (TMG). Cells induced for the lactose-PTS do not accumulate TMG if glucose or glucose analogues are transported by the mannose-PTS.[179,262,307] Inducer exclusion has mostly been attributed to competition for HPr(His ~ P) by affinity differences of HPr(His ~ P) for the sugar-specific EIIA proteins, but HPr(Ser ~ P) has also been implicated. The phenomenon of inducer expulsion is less understood, but in *Lc. lactis* the mechanism involves stimulation of a sugar phosphatase by HPr(Ser ~ P).[308] Figure 12 summarizes the central role of HPr in global regulation of carbon metabolism in LAB. The balance of the three different forms, HPr, HPr(His ~ P), and HPr(Ser ~ P), reflects the physiological state of the cell (the double phosphorylated form HPr(His ~ P)(Ser ~ P) also exists,

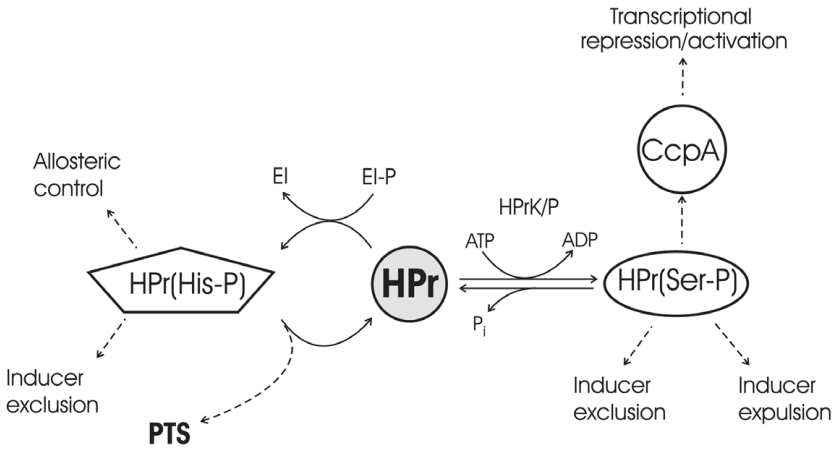

Figure 12 Schematic representation of the central role of HPr in the global regulation of carbon transport and metabolism. See text for further details. PTS, phosphoenolpyruvate : sugar phosphotransferase system.

but the physiological role is not understood). The different forms exert their functions through other global regulators, such as CcpA, and/or through allosteric control of different transporters, giving an enormous range of modulating and adjusting catabolic capacity.

Studies on the PTS system and the role of its components in carbon control in LAB have mostly been done in *Lc. lactis* and *Lb. casei*,[303] but very important contributions regarding HPr were also made in the heterofermentative species *Lb. brevis*.[309,310] CcpA and/or its gene have been found in all LAB investigated so far. One could therefore assume that the general picture outlined above is valid for all LAB.

Another angle in the studies of carbon metabolism and its regulation is to apply metabolic control analysis and metabolic optimization. Recently, such studies have been initiated for the primary metabolism in *Lc. lactis*, i.e., glycolysis.[311] In an elegant series of studies, where enzyme activities are modulated in small steps by a genetic method using artificial promoters,[312] the metabolic control of glycolysis was investigated (reviewed in Ref. 313). These studies have to some degree questioned the key controlling role over glycolysis attributed to glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[199,314] since changing GAPDH levels in growing cells did not alter the glycolytic flux or change the mode of fermentation from homolactic to mixed acid.[313,315] Further studies along these lines will be very valuable for understanding how the primary metabolism in LAB is controlled and regulated.

Genome sequencing will give another dimension to research of the complex networks that regulate metabolism. At the time of this writing, only two LAB genome sequences have been published,[18,19] but more than 30 are underway, some of them already completed but not yet published.[20] For some species more than one strain will be sequenced. This will be important in defining the background for differences in phenotype and physiology. The analysis of the *Lc. lactis* genome, the first completed among LAB, is the most advanced. Attempts are being made to build a regulatory network based on predictions of regulators from the annotated sequence, mutagenesis, phenotypic

studies, and postgenome methodologies such as two-dimensional gel electrophoresis (proteom analysis) and DNA microarrays (transcriptome analysis).[316] An estimate of 111 regulators were putatively identified from the sequence, but only 18 have been characterized. A further 34 were proposed a function, but 59 were not. Some of these were unique for *Lc. lactis.* The author acknowledges the large amount of work needed, including classical biology and physiology, before a more complete picture of the regulatory network can be established.

VII. CONCLUDING REMARKS

The aim of this review on lactic acid bacteria has been to indicate what they are (general description), who they are (classification), what they do (metabolism), and how they do it (metabolism, energetics, and transport). This has not been an easy task. LAB comprise a very diverse group of organisms, which have sufficient characteristics in common that some generalizations can be made. The overall view I would like to pass on is that LAB are more than just lactic acid producers. Lactic acid production is merely a reflection of an underlying metabolism, which is far more complex and, most importantly, more adaptive than one could imagine. The results of genome sequencing of LAB will certainly strengthen this view and, to some extent, have already done so. The second LAB genome to be completed, *Lb. plantarum* strain WCFS1, revealed a large region in the chromosome containing genes encoding several nutrient utilization systems and extracellular functions. This cluster of genes has been termed a "lifestyle adaptation region".[19]

LAB are perfectly adapted to environments rich in nutrients and energy sources. They have, therefore, dispensed with biosynthetic capability. Apparently genetic material for biosynthesis is still present, as shown for the sequenced *Lb. plantarum* strain mentioned above.[19] However, the nutrient requirements in minimal media[317] are greater than the sequence implicates, indicating that some of the biosynthetic genes are not expressed. The reasons for this are not known, but early studies showed that mutagenesis can render some lactobacilli prototrophic for some amino acids.[250] However, if these mutants are returned to a rich medium, they readily revert to the auxotrophic state. This may have evolutionary implications, but more important, it shows that there has been a strong selection for cells that are committed to life in rich environments. These environments are of course excellent for supporting growth of other microorganisms. LAB have therefore developed strategies to efficiently compete with these organisms. One important strategy is acid production and acid tolerance.[318] This may be why the term LAB arose. A group of bacteria were isolated from similar niches and turned out to be lactic acid producers. It was natural to group them together. Another possibility is the following: the commitment to life in rich environments demands a simple, but effective way of outcompeting other microorganisms. Solution: acid production! Therefore, we are left with, in reality (phylogenetically), a group of organisms that are diverse but physiologically similar, since they are specialized for nutrient-rich environments (limited biosynthetic ability) and their metabolism is aimed at acid production.

Although the reasoning above may be somewhat oversimplified, it is clear that the classification problems that always have been evident with regard to LAB may stem from an (historical) overemphasis on a few characteristics. We now have the means to determine natural relationships more objectively and more accurately than ever before. These relationships can probably be assessed more easily than defining common phenotypic characters for a particular natural group. In future classification of bacteria, in general,

it will be necessary to do "reverse phylogenetics," i.e., first define the natural relationships among bacteria with, e.g., rRNA sequencing and then search for the unifying phenotypic characteristics. Interestingly, some approaches in this direction have been taken with regard to LAB. Some enterococci are known to be able to synthesize cytochromes in the presence of heme. The study by Meisel et al.,[35] in which it was shown that a species of *Carnobacterium* (in the study designated *Lb. maltaromicus*) can synthesize cytochromes in the presence of heme, was done with the phylogenetically close relationship between carnobacteria and enterococci in mind. The authors (correctly) suspected that carnobacteria could be similar to enterococci because of a phylogenetic relationship.

The general metabolism and physiology of LAB reflect their adaptation to niches rich in nutrients. They have developed very efficient transport systems, which enable them to quickly take up the necessary solutes. Their extreme saccharolytic nature is another example. Genome sequencing has confirmed this picture. Again, the genome of *Lb. plantarum* can serve as an example as it revealed an impressive 25 complete sugar PTS systems.[19] However, as I have tried to emphasize, LAB have developed systems that will allow them to derive more energy from a rich medium than just carbohydrate. One of these systems involves the "cofermentations" that have been mentioned several times. By using a substrate, otherwise nonfermentable, as an electron acceptor during carbohydrate fermentation, they indirectly derive some energy from that substrate that otherwise would be lost. Electrogenic precursor/product exchange, e.g., malate and citrate metabolism, essentially serve the same purpose.

As indicated above and in Sec. III, Orla-Jensen's concept of LAB being a "great natural group" may not be entirely correct. However, the term lactic acid bacteria will probably be used in the foreseeable future, since it is useful to describe a group of organisms that have many physiological properties in common and, as a generalization, have similarities in their ecological behavior.

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and precision, as they did thousands of years ago. We hope that this volume convinces the reader that new and novel applications based on a better understanding on the potential of lactic acid bacteria in biotechnology, and especially of their role in promoting health and combating disease, are emerging. The potential significance more than justifies multi-disciplinary research in this field, with targets in both food and feed development and promoting human and animal health and well-being.

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2

Bifidobacteria and Probiotic Action

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The medical world has long been interested in the nutrient properties of yogurt. The theory of Metchnikoff,[1] which holds that milk fermented with *Lactobacillus* has a favorable influence on the endogenous intestinal flora, was challenged in 1915 by Rahe, who demonstrated that these microorganisms do not survive passage through the stomach and small intestine. Subsequently, numerous studies have been carried out on *Lactobacillus*. The frequently contradictory findings are due to the unreliability of the methods for isolating and identifying bacteria from stools.

Nutritionists subsequently turned their attention to other microorganisms. According to Gurr et al.,[2] "the microorganisms with the best chance of passing through the stomach and small intestine and colonizing the medium are those endogenous to the species consuming the fermented product." Research has been focused on the genus *Bifidobacterium*, which, unlike the bacteria of yogurt, which are not obtained from human ecosystems, are isolated from animals and humans.

The probiotic effects of *Bifidobacterium*, already alluded to when they were first discovered in 1899,[3] were demonstrated by Manciaux in 1958.[4] The therapeutic properties of this genus of bacterium led the Japanese to introduce it to their diet.[5–7] Since 1986, the traditional microflora of yogurt—*Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*—have been enhanced by a third bacterium belonging to the genus *Bifidobacterium* and sometimes associated with *Lactobacillus acidophilus*. This new product with pleasant organoleptic qualities has aroused considerable interest from consumers, who were soon followed by dairy industrialists and medical teams.

Note: This chapter is reprinted from the previous edition.

I. THE BIFIDOBACTERIA: DISCOVERY AND HISTORY

In 1899 at the Institut Pasteur, Tissier observed and isolated from stools of infants a bacterium with a very unusual and hitherto unknown Y-shape. Where to place this bacterium within the classification system was then addressed.

At the beginning of the century, taxonomy was based entirely on morphological criteria, and Tissier[3] named this bacterium *Bacillus bifidus communis*. In Italy at about the same time Moro[8] discovered in similar conditions a bacterium different from that of Tissier, which he identified as belonging to the genus *Lactobacillus*. Despite the differences between these two bacteria, Holland[9] proposed a common name, *Lactobacillus bifidus*, which was to develop and gain precision as time passed in parallel with the progress in biology.

Orla-Jensen,[10] using new methods, was responsible for a decisive shift in the direction of the history of taxonomy. The classification and identification of microorganisms, which had hitherto been based entirely on their morphology, henceforth took into account new criteria: the physiology, nutritive requirements of the energy metabolism, and above all metabolic and enzymatic characteristics of the organism. Thus, in 1967 De Vries and Stouthamer[11] demonstrated the presence in bifides of fructose-6-phosphate phosphoketolase (F6PPK) and the absence of aldolase and glucose-6-phosphatase dehydrogenase, two enzymes found in the lactobacilli. They therefore concluded that the classification of the bifidobacteria in the genus *Lactobacillus* is not justified.

Two trends were distinguished: the French school, which was for the separation of the genera *Lactobacillus* and *Bifidobacterium* to combine all bifid bacteria under the single classification of *B. bifidum*,[9,10,12–14] and the Anglo-Saxon school, which preferred to integrate the bifidobacteria in the genus *Lactobacillus*. Table 1 summarizes the various names proposed for this bacterium since its discovery[15] (see Table 8 for species isolated to date).

The advent of chemotaxonomy in the 1960s marked the beginning of another period in bacterial taxonomy. Research into the biochemistry of the prokaryotes has shown that analysis of the cell constituents could become an essential tool in the classification and identification of bacteria. The development of instruments of analysis made it possible to obtain accurate and reproducible data, minimize errors in individual research, and eliminate subjective judgments.[16] In 1965, with progress in molecular genetics, the teams of Sebald et al.[17] and Werner et al.[18] showed that the percentage of guanine + cytosine (G + C%) in the DNA of *Bifidobacterium* differed from that of *Lactobacillus*, *Corynebacterium*, and *Propionibacterium*. In 1974, the 8th edition of *Bergey's Manual of Determinative Bacteriology* recognized *Bifidobacterium* as a genus in its own right consisting of 11 species.[19] Today, this genus, which belongs to the Actinomycetaceae family,[20] includes 24 species, which are grouped according to their ecological origin: 15 are isolated only from animals, and 9 colonize the natural cavities of humans.[21]

II. MORPHOLOGY

The bacteria of the genus *Bifidobacterium* present a globally bacillar form, show gram-positive staining, and are immobile and nonsporulate.

These rods, with an irregular outer wall, are usually concave, and their extremities are generally swollen to form "lumps," which may have certain ramifications. It is,

Table 1 Chronology of the Taxonomy of *Bifidobacterium*

Name	Author	Year
Bacillus bifidus	Tissier	1900
Bacteroides bifidus	Castellani and Chalmers	1919
		1923–1934
Lactobacillus bifidus	*Bergey's Manual*, eds. 1–4	1920
Bifidobacterium bifidum	Holland	1924
Bacterium bifidum	Orla-Jensen	1927
Tisseria bifida	Lehmann and Neumann	1929
Nocardia bifida	Pribram	1931
Actynomices bifidus	Vuillemin	1934
Actinobacterium bifidum	Nannizzi	1937
Lactobacillus acidophilus var. *bifidus*	Puntoni	1938
	Weiss and Rettger	
Lactobacillus parabifidus		1938
Bifidobacterium bifidum	Weiss and Rettger	1938
Lactobacillus bifidus	Prevot	1939–1957
Cohnistreptothrix bifidus	*Bergey's Manual*, eds. 5–7	1944
Corynebacterium bifidum	Negrovi and Fischer	1949
Lactobacillus bifidus	Olsen	1950
Lactobacillus bifidus var. *pennsylvanicus*	Norris et al.	1953
		1957
Five groups of bifidus bacteria	György	
Description of human species	Dehnert	1963
New animal species	Reuter	1969
New animal species	Mitsuoka	1969
New animal species	Scardovi	1972
Creation of the genus *Bifidobacterium* constituted by 11 species	Holdemann and Moore *Bergey's Manual*, ed. 8	1974

Source: From Ref. [15].

however, not unusual to encounter more rounded shapes as well as very long or short bacilli of varying widths. Gram staining reveals a frequently irregular distribution of chromatin, which often accumulates in the bifurcations or lumps.[22,23] However, this polymorphism cannot be assimilated to degeneration since these forms can generate the initial forms once more.[22] It would appear rather that the composition of the culture medium is responsible for these V-, Y-, or X-shaped forms encountered in the genus *Bifidobacterium*.

Several medium constituents may influence the shape of these bacteria:

The concentration of *N*-acetylglucosamine, involved in the synthesis of peptidoglycan (Fig. 1), affects the shape of *B. bifidum* var. *pennsylvanicus*.[24]
Various amino acids (alanine, aspartic acid, glutamic acid, and serine).[25]
Ca^{2+} ions.[26–28]

The lower the levels of *N*-acetylglucosamine and amino acids, the more highly branched are the shapes. In contrast, in a favorable medium the bacilli are longer.[22]

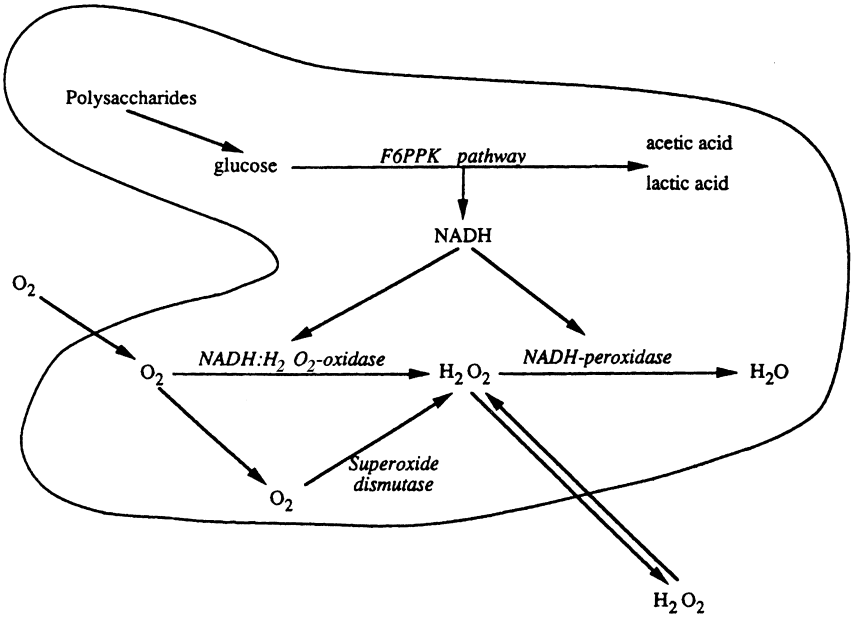

Figure 1 Oxygen dissimilation in *Bifidobacterium.*

III. PHYSIOLOGY

A. Respiratory Type

The bifidobacteria are strictly anaerobic microorganisms. However, the degree of tolerance of oxygen depends on the species and culture medium.[29]

Three types of responses are observed during the switch from anaerobiosis to aerobic conditions:

- Aerobic growth without the accumulation of H_2O_2: a strain of *B. bifidum* which is relatively aerotolerant, forms small quantities of H_2O_2 by NADH oxidation. The absence of H_2O_2 seen in liquid aerobic culture devoid of catalase of NADH peroxidase activity can be explained by an unknown peroxidase system, which could destroy the H_2O_2.
- Limited growth with the accumulation of H_2O_2, the accumulation of hydrogen peroxide is considered to be toxic for the key enzyme in the sugar metabolism of *Bifidobacterium*: fructose-6-phosphate phosphoketolase (F6PPK).[15]
- No growth without the accumulation of H_2O_2: the strains tested require a low redox potential for growth and fermentation.

In the presence of CO_2, the sensitivity to oxygen varies considerably depending on the strain. Among the strains able to develop in the presence of oxygen, some remain catalase negative, others become catalase positive, and for others still the presence of catalase is linked to the presence of hemin in the medium.[20]

A study of the absorption of oxygen by five strains of *Bifidobacterium* of human origin has shown that the partial pressure of oxygen falls in the medium during the multiplication of these strains. The endogenous absorption of oxygen is linked to

the presence of NADH oxidase. It takes place even in the absence of glucose and appears to depend directly on the quantity of polysaccharides accumulated in the cells. Furthermore, all strains accumulated hydrogen peroxide, which is subsequently reduced by NADH peroxidase, but the activity of this enzyme varied depending on the strain investigated. The strains most sensitive to oxygen had low NADH peroxidase activity, resulting in an accumulation of toxic hydrogen peroxide. Another possibility would be the prevention of multiplication by the presence of active oxygen such as superoxide. These conclusions are summarized in Fig. 1.[30]

The mutants of some strains identified at the time as *B. bifidum* characterized by the loss of strictly aerobic character have been isolated,[22,31] but these early studies should be repeated in view of the difficulty in identifying species of *Bifidobacterium* at the time these studies were performed.

B. Temperature and pH

The optimum temperature for the development of the human species is between 36 and 38°C. In contrast, that for the animal species is slightly higher, about 41–43°C and may even reach 46.5°C. There is no growth below 20°C, and the bacteria of this type have no thermoresistance above 46°C: *B. bifidum* dies at 60°C.[15]

The initial optimum growth pH is between 6.5 and 7.0. No growth can occur below 5.0 or above 8.0.[20]

IV. METABOLISM

A. Sugar Metabolism

In the genus *Bifidobacterium* hexoses are degraded exclusively and specifically by the fructose-6-phosphate pathway described by Scardovi and Trovatelli.[32] Aldolase and glucose-6-phosphate dehydrogenase are absent, whereas fructose-6-phosphate phosphoketolase is found[11] (Fig. 2).

B. Metabolites

The fermentation of two moles of glucose leads globally to three moles of acetate and two moles of lactate. In reality, pyruvic acid can be broken down along two pathways: the first is the reduction of the pyruvate to form L(+)-lactate by L(+)-dehydrogenase (E.C. 1.1.1.27), an enzyme controlled by fructose-1,6-diphosphate.

The second pathway involves the splitting of pyruvate by phosphoroclastic enzyme to form formic acid and acetyl phosphate, a portion of which is subsequently reduced to form ethyl alcohol and so regenerate NAD. However, tests carried out to detect phosphoclastic enzyme have been unsuccessful.[33]

The proportions of the final fermentation products vary considerably from one strain to another and even within the same species.[33] Small quantities of succinic acid are produced by some strains, and a small amount of CO_2 may be produced during the degradation of gluconate.[20]

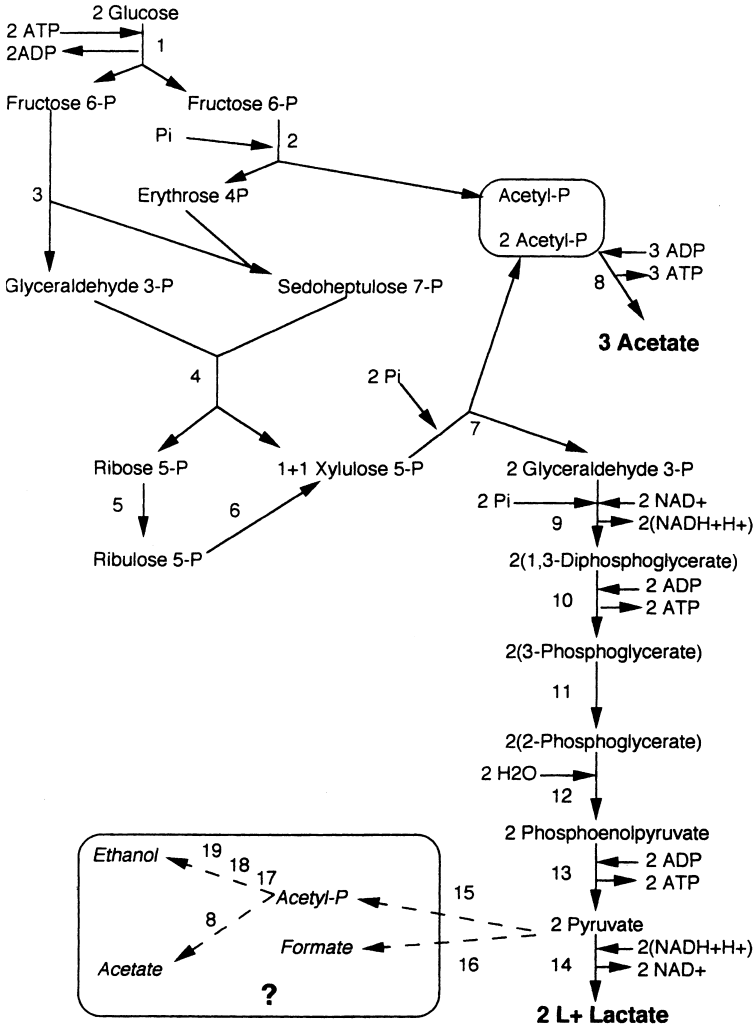

Figure 2 Metabolic pathway of *Bifidobacterium.* 1 = hexokinase and glucose-6-phosphate isomerase; 2 = fructose-6-phosphate phosphocetolase; 3 = transaldolase; 4 = transketolase; 5 = ribose-5-phosphate isomerase; 6 = ribulose-5-phosphate epimerase; 7 = xylulose-5-phosphate phosphocetolase; 8 = acetate kinase; 9 = homofermentative pathway enzymes; 10 = L(+) lactate dehydrogenase; 11 = phosphoroclastic enzyme; 12 = formate dehydrogenase (EC 1.2.1.2); 13 = alcohol dehydrogenase (EC 1.1.1.1).

C. Enzymes

The final fermentation products are formed by the sequential action of transaldolase, transketolase, xylulose-5-phosphate phosphoketolase, and enzymes belonging to the Embden-Meyerhoff-Parnas pathway, which act on glyceraldehyde-3-phosphate.

The characteristic enzyme of sugar metabolism by the genus *Bifidobacterium* is fructose-6-phosphate phosphoketolase (F6PPK, EC 4.1.2.22). This enzyme, which is specific to the genus, is absent in the anaerobic bacteria which could be morphologically

confused with the bifidobacteria, that is, *Lactobacillus*, *Arthrobacter*, *Propionibacterium*, *Corynebacterium*, and Actinomycetaceae.[32]

Biavati et al.[35] have demonstrated, using electrophoresis in starch gel (zymogram) followed by comparison of electrophoretic metabolism, three different types of this enzyme depending on the ecological source of the strain: mammalian, bee, or human.[36] The F6PPKs of *B. globosum* (animal type) and *B. dentium* (human type) have been purified.[37]

Using the same method, 14 isoenzymes of transaldolase (EC 2.2.1.2) and 29 isoenzymes of 6-phosphogluconate dehydrogenase (6PGD) (EC 1.1.1.44) have been identified.[37] Transaldolase is an apparently essential enzyme characteristic of the fructose-6-phosphate shunt, but 6PGD is apparently nonfunctional in the bifidobacteria, at least in cells cultured on glucose, which are generally deficient in detectable glucose-6-phosphate dehydrogenase.[32]

Some research has been carried out on other less characteristic enzymes:

1. Miura et al.[38] determined the following activities by HPLC:
   Arylsulfatase (R-sulfate + $H_2O \rightarrow R-OH$ + sulfate).
   β-glucuronidase (R-glucuronide + $H_2O \rightarrow R-OH$ + glucuronic acid) and β-glucosidase (hydrolysis of the aryl- or alkyl-β-glycosides).
   Galactokinase (EC 2.7.1.6) (galactose + ATP $\rightarrow$ galactose-1-phosphate + ADP) of *B. bifidum* purified and characterized after growth on galactose.[39]
2. Tochikura et al.[40] purified β-D-galactosidase from *B. longum* (lactose + $H_2O \rightarrow$ galactose + glucose).
3. Desjardins and Roy[41] used API ZYM systems to determine 22 strains of human origin that possess α- and β-galactosidases and α-glucosidase activities. In contrast, β-glucosidase has not been detected in either *B. bifidum* or *B. longum*. This method can, however, be used in a preliminary study. These studies were confirmed in the same year by Chevalier et al.[42] β-Glucosidase, β-glucuronidase, and *N*-acetylglucosaminidase activities have also been demonstrated.

D. The Vitamins Produced

Deguchi et al.[43] were interested in the synthesis of six vitamins by *Bifidobacterium* of human origin: thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), folic acid (B_9), cyanocobalamine (B_{12}), and nicotinic acid (PP). Five of these vitamins (with the exception of riboflavin) are synthesized by most of the strains examined, and a large proportion of each (B_6, B_9 and B_{12}) is excreted. These authors also note that with regard to thiamine, folic acid, and nicotinic acid, *B. bifidum* and *B. infantis* are good producers, whereas *B. breve* and *B. longum* release small quantities and *B. adolescentis* does not synthesize any of these vitamins.

The production of vitamins B_2 and B_6 by *B. longum* is exceptional. *B. breve* and *B. infantis* are characterized by a high level of production of vitamins PP and H, respectively. The results are shown in Table 2.

E. Nutrient Requirements

1. Nitrogenous Matter

Most strains of *Bifidobacterium* are able to use ammonium salts as their only source of nitrogen.[44] However, *B. suis*, *B. magnum*, *B. choerinum*, and *B. cuniculi* develop only

Table 2 Vitamin Production by *Bifidobacterium*

	B. breve	*B. infantis*	*B. longum*	*B. bifidum*	*B. adolescentis*
Thiamine (B_1)	+	+++	+	+++	+
Riboflavin (B_2)	+	+	+++	++	+
Pyridoxine (B_6)	++	++	+++	+	++
Folic acid (B_9)	+	+++	+	++	+
Cobalamine (B_{12})	+	++	+++	+	+
Ascorbic acid (C)	++	++	+++	++	+
Nicotinic acid (PP)	+++	+++	+	+++	+
Biotin (H)	++	+++	++	++	++

in the presence of organic nitrogen. In vitro and in the absence of any organic source of nitrogen, the bifidobacteria may synthesize large quantities of amino acids. *B. bifidum*, for example, produces alanine, valine, and aspartic acid and up to 150 mg/L of threonine.[45] According to Hatanaka et al.[46,47] the glutamine synthetase and glutamate dehydrogenase of the *Bifidobacterium* may be involved in the assimilation of nitrogenous compounds by these microorganisms.

2. Trace Elements

B. bifidum grows only in the presence of magnesium, manganese, and above all iron. Iron may be assimilated by *B. bifidum* in both oxidation forms depending on the acidity of the medium.[48–51]

Fe^{2+} ferrous iron is used at pH 5. Transport depends on a membrane ATPase, and its incorporation may be competitively inhibited by zinc.[48]

Fe^{3+} ferric iron is used only at neutral pH. Through the intermediary of ferro-enzymes, iron is involved in the production of acetic acid by *B. bifidum*.

3. Vitamins

It is impossible to draw up any rule for the genus *Bifidobacterium* with regard to vitamin requirements. Strains of human origin seem to need thiamine (B_1), pyridoxine (B_6), folic acid (B_9), cyanocobalamine (B_{12}), and nicotinic acid (PP) for their growth (Teraguchi et al., 1984).[43]

4. Growth Factors

Poch and Bezkorovainy[52] supplemented an entirely synthetic minimum base medium with growth factors in order to identify those essential to the development of the various species of *Bifidobacterium*. Only *B. adolescentis* and *B. longum* were able to develop in the unsupplemented medium. All the other species required the presence of growth factors of various types.

Bifidigenic Factors. In 1953, György[53] discovered a strain of *B. bifidum* (then known as *L. bifidus*) which was to develop only in the presence of human milk and more specifically in the presence of derivatives of *N*-acetylglucosamine[54–56] and showed soon afterwards that the strain *B. bifidum* Tissier required protein factors and not *N*-acetylated sugars for its development.

In fact, the species *B.bifidum* can be divided into two variants: the "A" variant or *B. bifidum*, which Tissier found in adult human beings, and the "B" variant or *B. bifidum*

var. *pennsylvanicus*, which György isolated from infants. These observations suggest that the various strains of the same species *B. bifidum* have differing nutritive requirements, *B. bifidum* var. appears to be insensitive to *N*-acetylglucosamine derivatives and to require protein factors in the same way as *B. longum* and *B. infantis*, whereas the "B" variant of *B. bifidum* requires the sugar factors from human milk in varying quantities depending on the strains.[57–59]

Most species of the genus *Bifidobacterium* are unable to develop in a totally synthetic medium and require complex biological substances such as bovine casein digestate, lactoserum of bovine milk, porcine gastric mucin, or yeast extract.[52,60]

These growth factors required for the development of *Bifidobacterium* are known as bifidigenic factors. We can now distinguish three main groups of bifidigenic factors which differ depending on the species with which we are concerned:[61] the BB factors (BFl, BF2, and glycoproteins) and the BI and BL factors (Table 3).

BB FACTORS. The factors BBa and BBb are characterized as the elements in human milk that do not lose their stimulant activity for *B. bifidum* var. a and *B. bifidum* var. b, respectively, heating or irradiation. The BBa factors are found mainly in yeast extracts, liver extracts, lyophilized milks, bovine casein hydrolysate, and porcine mucin,[23,62,63] whereas colostrums, human milk and rat milk,[64,65] human casein hydrolysates,[62] and porcine mucin[23] contain BBb factors (Table 3).

Three groups of natural BB factors can be distinguished.

1. *György's bifidus factor I or BF1*. This is factor BF1 found in milk and colostrum and in the form of gynolactose, which is active particularly on variant b. It would seem that the presence of an *N*-acetylglucosamine structure in the oligosaccharide structure is essential but not sufficient to the expression of bifidigenic activity.[66] In addition, *B. bifidum* var. *pennsylvanicus* has *N*-acetyl-D-glucosaminidase activity, which is considerably greater than that found for other bifidobacteria.[41]

   Native human casein[66] or its trypsin hydrolysate[63] consisting of glycoproteins may be effective versus *B. bifidum* var. b. The trypsin or chymotrypsin hydrolysis of native human κ-casein gives rise to fractions containing 60–70% of sugars such as galactose, glucosamine, and galactosamine, which are themselves active.

   The mucins (glycoproteins of mucus) are produced and secreted by the mucus cells of the salivary glands, the esophagus, the stomach, the small intestine, and colon. Their molecular weight exceeds one million daltons.[67] The mucins, which are the major constituents of mucus[68] consist of 70–80% sugar.[69] The oligosaccharide chains contain between 2 and 20 monosaccharide residues, which may be the following: galactose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid.

   These oligosaccharide chains are linked to peptide segments accounting for 20% of the weight of the molecule and consisting of more than 70% proline, serine, and threonine.

   Porcine gastrointestinal mucins and the meconium are an abundant source of BB factors. The activity of the meconium in vitro is 1.2–2 times greater than that of human milk.[53] Mild hydrolysis of the mucins give rise to oligosaccharides similar to those in human milk.[70,71]

Table 3 Characteristics of the Main Bifidigen Factors

Bifidigen factor	Species concerned	Source	Resistance			Active structure
			Heat	Ray.	Lyoph.	
BB BF1	*B. bifidum* var. b	Milk and colostrum Human casein hydrolysate Mucins	+	+		*N*-Acetylglucosamine glycoproteins
BF2	*B. bifidum* var. a	Casein hydrolysate	+	+		Nonglycosylated peptides
Glycoproteins	*B. bifidum* var. a *B. bifidum* var. b	Human milk and colostrum				Glucidic part
BI	*B. infantis*	Plant extracts Liver extracts Milk	±	±	−	Proteic part
BL	*B. longum*	Plant extracts Liver extracts Milk	−	−		Proteic different from BB factors

2. *BF2 factors*. Their nature has been described essentially by Raynaud[56] from a strain of *B. bifidum* var. a. They appear to consist of nonglycosylated peptides obtained by the action of a protease on casein.
3. *Glycoproteins*. The glycoproteins isolated from human colostrum and milk lactoserum appear to be effective versus both variants, and this type of activity appears to be related to their sugar fraction.[66]

BI AND BL FACTORS. The BI factor, which stimulates the growth of *B. infantis*, is destroyed by lyophilization, whereas the BL factor, which activates the growth of *B. longum*, is sensitive to heating and irradiation. These factors are abundant in many plant extracts as well as liver and milk extracts. The BI factors from human milk are of two types: thermo- and radiolabile BI and thermo- and radiostable BI. These factors are proteins, as are the BL factors of human milk.[59]

ACTIVE CONSTITUENTS OF THE BIFIDIGENIC FACTORS. The factors with general activity are hydrolysates of bovine casein and yeast extracts rather than human milk lactoserum. The other growth factors, human or bovine milk lactosera, porcine gastric mucin, and bovine albumin serum digestate, are active with regard to certain species only.[52]

In fact, the disulfide/sulfhydryl residues of κ-casein are important biologically active compounds responsible for this phenomenon in *B. bifidum* and *B. longum*. The growth-promoting activity resides in the κ-casein portion and not in the carbohydrate portion after trypsin digestion. It appears that the combination of disulfide/sulfhydryl residues with something else is the basis of the microbial growth-promoting activity in hydrolysates of casein, porcine gastric mucin, and yeast extract.[72]

ROLE OF THE BIFIDIGENIC FACTORS AND CONCLUSION. In vivo, the administration of a dairy-based food supplemented with BF1 or BF2 factor to infants restores the bifidum flora partially.[73] These early studies should, however, be repeated on the basis of the recent understanding of the taxonomy of *Bifidobacterium* and the biochemistry of the bifidigenic factors, which appear to be highly complex. The correlation between a given factor and a given species appears to be an important aspect of the study of the bifidigenic factors. It would be important to explore the specificity of these factors with regard to the species of *Bifidobacterium* that colonize the intestine, since the degree of difference between the collection strains and strains encountered in nature is doubtless considerable. Furthermore, the studies conducted were carried out in vitro or in vivo on axenic or monoxenic animals, and these require extrapolation to humans. What is the influence of the "bifidigenic" factors on the other bacterial genera of the intestinal flora and particularly of the human flora?

Lactoferrin. Lactoferrin and its three metal complexes (Fe, Cu, Zn) have a promoting effect on the growth of eight species of *Bifidobacterium*, five of human origin and three of animal origin, at the beginning of the logarithmic growth phase. Furthermore, these lactoferrin-metal complexes demonstrate an anti-bacterial activity versus *E. coli* and *Staphylococcus aureus*.[74]

Lactulose and Lactitol. Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) is not metabolized by human or animal species. Lactulose, which is not detected in raw milk, is present in dairy products subjected to heat treatment. Manciaux[4] reported that Petuely, in the 1930s, isolated lactulose from human milk. In vivo, lactulose can increase the development of *B. bifidum*. However, this factor is not active in vitro and is not present in the free state in mother's milk. According to Petuely,[75] its action is

due to the fact that it resists better than lactose degradation by lactases in the digestive tract and can therefore be used massively by the bifidobacteria. Lactulose is not used specifically by the bifidobacteria and may be metabolized by other intestinal bacteria, as must be the case for the bifidigenic factors.[76] Lactitol is considered to be a bifidigenic factor with a less marked effect.[77]

Oligoholosides and Polyholosides. Raffinose, stachyose, and insulin (polyfructose) with molecular weights of less than 4500 are used only by *B. infantis* and not by other intestinal bacteria such as *E. coli*, *L. acidophilus*, and *S. faecalis*. Oligosaccharides higher than the trisaccharide of inulin and the tri- to pentasaccharides of dextran are also metabolized specifically by *B. infantis*. In contrast, the oligosaccharides of amylose and cellulose are not specific to *B. infantis* and *B. breve*.[76]

Fructooligosaccharides. These polymers of fructose with a degree of polymerization of between 2 and 35 have a stimulant effect on the growth of bifidobacteria.[78] They are metabolized by bifidobacteria and also by other types of bacteria and are not degraded by human digestive enzymes nor generally by undesirable microorganisms within the digestive tract. The most important source of fructooligosaccharides is the Jerusalem artichoke tuber.[77] Today it is easier to prepare a similar substance by an enzymatic route from sugar[78,79] than to purify fructooligosaccharides from natural sources.[80]

V. RESISTANCE TO ANTIBIOTICS

Knowing which antibiotics the bifidobacteria are resistant to is of interest for two reasons:

1. It offers the possibility of maintaining the bifidobacteria in the digestive tract without aggression, particularly during antibiotic treatment.
2. It makes it possible to incorporate antibiotics as selective agents in culture media for the isolation of bifidobacteria from complex flora derived from medical or dietary samples.

The sensitivity of bifidobacteria has been the subject of little research, and the works done before the publication of an international standard are difficult to compare because experimental conditions vary. However, we can accept the following points: most bifidobacteria are resistant to numerous antibiotics—notably to nalidixic acid, gentamicin, kanamycin, metronidazole, neomycin, polymyxin B, and streptomycin—but the sensitivity of the species varies from 10 to 500 or more μg antibiotic/mL[73] in *B. bifidum*.[81,82] In contrast, ampicillin, bacitracin, chloramphenicol, clindamycin, erythromycin, lincomycin, nitrofurantoin, oleandomycin, penicillin G, and vancomycin strongly inhibit most species.[20] Sensitivity to tetracycline varies from one species to another and even from one strain to another (Matteuzi, unpublished; Ref. 73).

VI. CULTURE MEDIA AND CULTURE PARAMETERS

Three types of medium have been designed for the isolation, culture, and characterization of bifidobacteria: complex, semisynthetic, and synthetic media.

A. Complex Culture Media

These richly varied media are prepared from liver or meat extracts, a wide range of peptones, yeast extract, tomato juice, horse blood, or human milk and permit the growth of as many strains of *Bifidobacterium* as possible. In addition, they are supplemented with substances with a low redox potential: cysteine, cystine, ascorbic acid, or sodium sulfite.[15]

A wide range of complex culture media have been proposed in recent years. We will consider the following:

BL agar medium, described by Ochi et al.[83] and then by Mitsuoka et al.[5,84] and finally slightly modified by Teraguchi et al.,[85] is considered to be the optimum culture medium for the detection of bifidobacteria.

Scardovi's tryptone phytone yeast medium (TPY)[20,21] can be used for the culture and isolation of bifidobacteria but also of other lactic bacteria from all habitats.

Mention should also be made of the YN-6 medium[86] and YN-17 medium.[87] These media are not very efficient since YN-6 medium inhibits some species of *Bifidobacterium* but allows other genera to develop,[88] whereas YN-17 medium inhibits some of the bifidum population.[89]

B. Semisynthetic Culture Media

Complex constituents of known composition are included in these media. We will note particularly the following:

Tomarelli's medium[90] for the culture of *B. bifidum*

Norris's medium,[31] which is a modification of the Tomarelli medium

György's medium,[65] which is also a modified Tomarelli medium.

C. Entirely Synthetic Culture Media

All the constituents of these media are chemically defined.

Petuely[75] was the first to propose a synthetic culture medium.

Hassinen medium.[44]

Gyllenberg modified the Petuely medium.[91]

Tanaka and Mutai medium.[92]

Ueda et al.[51] developed a synthetic medium for the culture of the ES5 strain of *B. bifidum.*

Poch and Bezkorovainy medium.[52]

D. Selective Culture Media

The media listed above are efficient for the maintenance of pure strains but are less effective for isolating them from complex flora since they often permit the growth of other genera.

Since the physiological requirements of bifidobacteria are extremely varied, it is difficult to define a selective medium appropriate for all species.[20] Since the recent enthusiasm for incorporating bifidobacteria in fermented dairy products, several selective media have been proposed in order to differentiate between *Bifidobacterium* and other lactic bacteria and to isolate bifidobacteria from the intestinal flora.

Initially, ascorbic acid and sodium azide were used as selective substances.

Beck[93] isolated *Bifidobacterium* on a medium containing added bifidigenic growth factors (*N*-acetyl-D-glucosamine). The relatively low pH of this medium (5.8) and anaerobiotic culture makes it possible to eliminate most enterobacteria.

Chang et al.[94] modified the MRS agar medium, the new medium containing cysteine, azide, and China ink in order to isolate numerous species of *Bifidobacterium.*

Matteuzzi et al.[82] suggested the addition of 80 μg of kanamycin/mL to the medium. However, intraspecific variations of resistance are so great that the isolation of unknown strains with a medium of this type would be unreliable.

Ushiima et al.[95] were able to selectively isolate *B. adolescentis* from a complex gastric flora by using selective agents: polymyxin, propionate, and linoleate.

Sonoike et al.[96] took into account the fact that bifidobacteria are able to metabolize carbohydrates such as fructo- and galactosyl-oligosaccharides. Twenty-two species of *Bifidobacterium* are able to develop on a medium containing *trans*-galactosylated oligosaccharides as a carbon source.

Munoa and Pares[89] attempted to quantify *Bifidobacterium* from water on a new selective medium: *Bifidobacterium* iodoacetate medium 25 (BIM-25). This medium is a reinforced clostridial medium (RCM) containing added antibiotics (nalidixic acid, polymyxin B, kanamycin), iodoacetic acid, and 2,3,5-triphenyltetrazolium chloride (TTC). Iodoacetate, which inhibits glyceraldehyde-3-phosphate dehydrogenase, considerably reduces the growth of nonbifidum colonies. TTC makes it possible to differentiate between *Bifidobacterium* and other species since the bifidobacteria develop in large white colonies.

Poch et al.[52] supplemented a synthetic base medium with various substrates in order to identify the growth factors necessary for each species of *Bifidobacterium*. The base medium was similar to Norris's medium.[97]

Mitsuoka used propionate as one of the selective agents added to BL agar medium for the selective counting of intestinal bifidobacteria. The BS agar medium thus obtained is not entirely satisfactory for the detection of bifidum in the stools.

Beerens[98] proposed a selective and elective medium by adding 5 g/L of propionic acid to Colombia agar and adjusting the pH to 5.5. This medium is relatively commonly used.

E. Culture Parameters

The appearance of the colonies of *Bifidobacterium* cultured on agar medium under anaerobic conditions may vary in function of the medium and the species used, but also within a given species. In general, the colonies formed are round, dull or glossy and of variable diameter, but Scardovi[20] and Boventer[99] distinguished two differing types of colony for *B. bifidum*. Some colonies were smooth, convex, white, and shiny, whereas other colonies were rough, with uneven edges and map.

VII. COMPOSITION OF THE WALL

The main constituent of the bacteria wall of the genus *Bifidobacterium* (gram-positive) is mucopeptide, peptidoglycan, or murein. This is macromolecule consisting of linear

polysaccharide chains which are linked with each other by tetrapeptide bridges associated with peptides. The polysaccharide chain consists of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). The tetrapeptides are linked to the NAM residues and to each other through the intermediary peptides (Fig. 3). The amino acids constituting the various peptides are alanine, glutamic acid, ornithine or lysine associated with one or two of the following: glycine, serine, aspartic acid, or threonine.[100,101]

The amino acids may be the same or may differ from one strain to another, but even if they are the same, their sequence within the tetrapeptide and their types of cross-linkage may vary,[102] i.e., may consist of a simple amino acid, a dipeptide, or even a tripeptide. *B. longum*, for example, possesses an ornithine-type tetrapeptide and the link peptide is L-Ser–L-Ala–L-Thr–L-Ala.[15] This macropeptide is covalently linked to other macromolecules, such as (a) polyosides: glucose, galactose, and rhamnose comprising the polysaccharide portion of the wall, and (b) teichoic acids, which are polymers of glycerol phosphate. These teichoic acids are attached to the NAG-NAM skeleton of the peptidoglycan.

VIII. CHARACTERISTICS OF THE GENOME

A. DNA Base Composition

The G + C% of the bacteria of the genus *Bifidobacterium* is between 57.2 and 64.5%.[17,20]

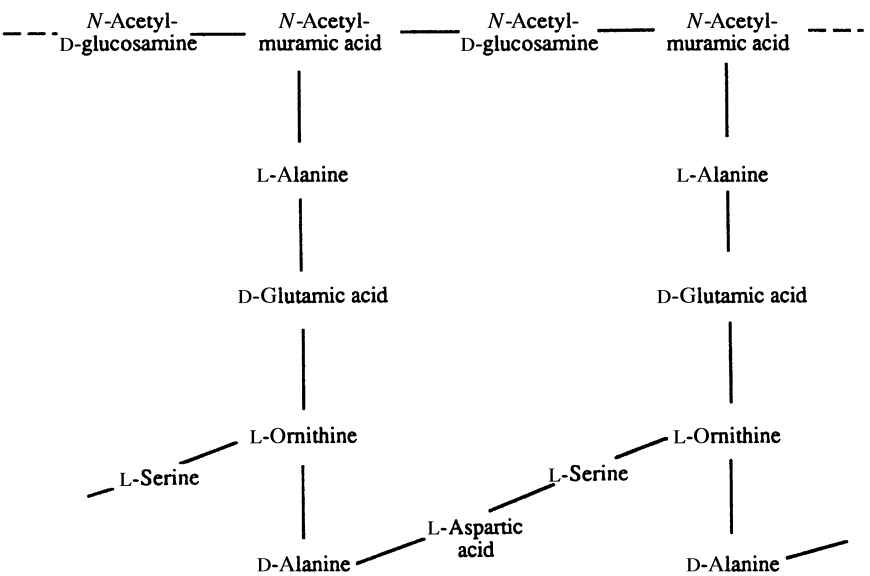

Figure 3 Peptidoglycan structure of *Bifidobacterium bifidum*. (From Ballongue, 1989.)

B. Plasmids

Of the 24 species of *Bifidobacterium*, only 5 species have plasmids:[103,104]

1. *B. longum* contains 13 model plasmids (1.25–9.5 MDa).[104,105]
2. *B. globosum* contains a plasmid belonging to each of the three molecular weight categories (13.5, 24.5, and 46 MDa).
3. *B. asteroides* has 14 types of plasmids (1.2–22 MDa), which are structurally very varied. (B. Sgorbati, unpublished).
4. *B. indicum*: 60% of the strains isolated contain a single 22 MDa plasmid.
5. *B. breve*: Iwata et al.[106] have demonstrated plasmids in 40% of the strains of this species, even though Sgorbati et al.[103] had not found any plasmid in *B. breve*.

In some strains these plasmids may be temperate phages, but this appears to be unusual.[105] Very curiously, *B. infantis*, which constitutes a continuum with *B. longum*, as we shall see below,[57,107] has no plasmid. No phenotypic characteristic has so far correlated with the presence of plasmids.[103]

IX. *BIFIDOBACTERIUM* ECOLOGY

Of the 24 species of *Bifidobacterium* so far recognized, 9 are isolated essentially from humans: *B. bifidum*, *B. longum*, *B. infantis*, *B. breve*, *B. adolescentis*, *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum*, and *B. dentium*.

A. Implantation in the Neonate

It is generally admitted that until the time of birth the fetus is surrounded by a completely sterile environment. After birth, the digestive tract is rapidly colonized by bacteria.[108–110] Forty-eight hours after birth, the colon contains 10^9–10^{10} bacteria per gram of stools consisting mainly of enterobacteria, staphylococci, and streptococci.[109–111]

The bifidobacteria appear only between day 2 and day 5[109] and become dominant (10^{10}–10^{11} per gram of stools) barely one week after birth. They reach a level of 99% of the fecal flora, whereas the levels of other bacteria (*E. coli*, lactobacilli, enterococci) decline sharply by about 1000-fold.[112–115] Anaerobes such as *Bacterioides* and *Clostridium* and other putrefying bacteria are enormously reduced and may disappear.

1. Origin of Colonization

This rapid invasion of the sterile digestive tract at birth raises the question of the origin of the bacteria and more particularly of anaerobes such as *Bifidobacterium*, which survive only precariously in the atmosphere. Do the bacteria invade from the digestive tract from the mouth or from the rectum? Several studies tend to disprove the hypothesis that the digestive tract is colonized by the rectal route[116,117] and demonstrate that, on the contrary, bifidobacteria enter the body of the neonate by an oral route:

Kleinschmidt[118,119] detected *B. bifidum* in the upper segment of the digestive tract of a child operated due to the absence of an anal perforation.

Boventer[99,120] clearly showed that the implantation of *Bifidobacterium* in the digestive tract occurs by descending route, since the rectum remains sterile until colonization is complete.

Mutai and Tanaka[121] isolated and observed in the mouth of 23 neonates *Bifidobacterium*, *Propionibacterium*, *Bacteroides*, *Peptostreptococcus*, *Fusobacterium*, *Enterococcus*, *Lactobacillus*, and Enterobacteriaceae 10 minutes after birth via the genital tract, whereas following cesarian section only *Propionibacterium* and *Enterococcus* were isolated in 8 out of 9 neonates investigated.

Another weighty argument in favor of the hypothesis of colonization by the vaginal or fecal flora of the mother is the observation by many authors that invasion of the digestive tract of the neonate occurs much more rapidly after vaginal birth than after birth by cesarian section.[122] Mitsuoka, et al.[109] isolated bifidobacteria in 41% of vaginal births, whereas Bezirtzoglou[110] found them in 21% of infants aged 4 days and born by cesarian section (41% after 15 days).

2. Factors Influencing Colonization

In addition to the method of delivery, which we have just seen directly affects the speed of invasion by bifidobacteria, several other factors also influence colonization.

Prematurity. This is a cause of difficult implantation of *Bifidobacterium* due to the lack of receptors and/or endogenous substrates, whereas enterobacteria and *Bacteroides* readily to colonize the colon.[112–114,123,124]

Method of Feeding. Tests for bifidobacteria in mother's milk have always been negative,[110] with the exception of the findings of Mayer and Moser,[22] who isolated *B. bifidum* from the colostrum and milk of a woman before breast-feeding commenced.

INFLUENCE OF THE TYPE OF FEEDING ON THE COMPOSITION OF THE INTESTINAL FLORA. The effects of breast-feeding and bottle-feeding have been compared in numerous studies. At the beginning of the twentieth century, Tissier wrote that the flora of a neonate raised on the breast consisted entirely of *Bifidobacterium*, whereas lactobacilli were predominant in the flora bottle-fed infants. From subsequent research it has emerged that there is no difference in the qualitative distribution of species between these two types of feeding. The difference lies in the quantitative level in the proportion of *Bifidobacterium* and other species, with clearly higher levels of *Bifidobacterium* for children breast-fed who show high levels of *Bifidobacterium*, which are generally higher than those of enterobacteria and *Bacteroides* within the first week of life (see Table 4 and Fig. 4).[5,111,125–131]

The stools of a breast-fed child are also characterized by a granular appearance, slightly vinegary smell, and marked acidity (pH 48–5.2),[132] which is probably due to the abundance of *Bifidobacterium* and therefore to considerable acetate production.[109,132–134] In contrast, the stools of children fed artificially are more similar to those of adults (pH 6.4–7.0)[58] which indicates the presence of putrefying organisms.

Thus, in artificial feeding the fundamental difference lies in the maintenance of high levels of optional aerobic species (*E. coli* and streptococci) which initially colonized the digestive tract and the development of anaerobes (*Bacteroides*, *Clostridium*, *Eubacteria*, *Peptostreptococcus*, *Clostridia*, Enterobacteriaceae, *Streptococcus*).[135,136] *Bifidobacterium* appears fairly late and is found in lower proportions in the stools.[57]

Some authors,[137,138] on the contrary, note that breast-feeding does not increase the level of *Bifidobacterium* in the first few days after breast-feeding, the predominant popu-

Table 4 Comparison Between Fecal Flora[a] of Breast-Fed and Bottle-Fed Infants

Bacteria	Breast-fed infants	Bottle-fed infants
Enterobacteriaceae	8.6	9.5
Streptococcus	7.9	9.8
Staphylococcus	5.8	5.5
Lactobacillus	7.0	5.9
Bifidobacterium	10.7	10.0
Eubacteria	3.1	7.3
Bacteroidaceae	6.1	9.9
Peptococcaceae	2.4	7.9
Cl. perfringens	1.0	6.4
Clostridium	1.3	0.9
Veillonellae	5.8	5.9

[a] log cfu.

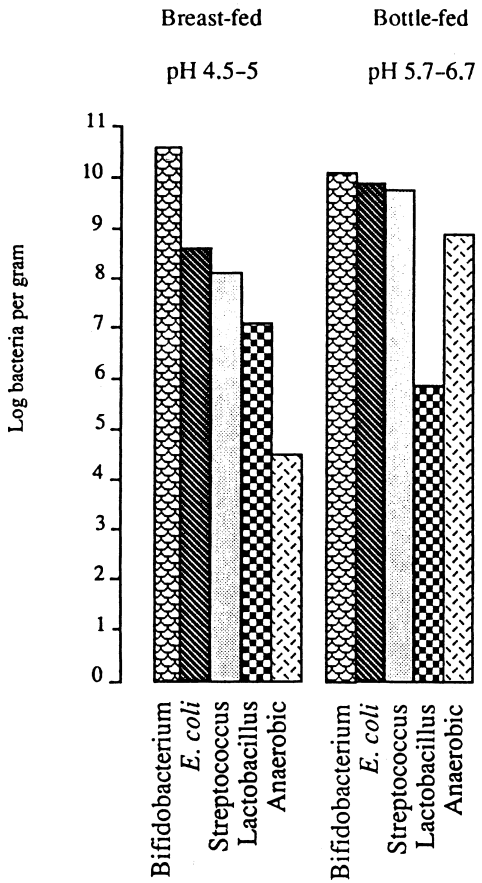

Figure 4 Comparison of fecal flora between breast-fed and bottle-fed infants.

lation consisting of enterobacteria and not of strictly anaerobic species. Benno et al.[131] note that in both types of feeding, *Bifidobacterium* constitutes the predominant genus (Fig. 5).

At weaning, a sudden change occurs in the fecal flora following the first bottle-fed[139] or solid food. In some children the bifidum flora falls sharply; in others, however, it remains stable. There is a remarkable proliferation of *Bacteroides*, *Eubacterium*, *Peptostreptococcus*, and *Clostridium*.[136] In all cases, the ratio of *Bifidobacterium* "putrefying flora" falls and is reversed. To date, no industrial dairy formula has made it possible to maintain the same equilibrium as that found during breast-feeding.

INFLUENCE OF THE TYPE OF FEEDING ON *BIFIDOBACTERIUM* SPECIES. The proportions of the various species of *Bifidobacterium* also vary with the type of feeding.[129] *B. bifidum* appears to be the dominant species during breast-feeding.[59,127,130] In contrast, in Italy, Biavati et al.[140] did not observe any change in the distribution of the species in breast- or bottle-fed infants:

B. bifidum	3%
B. longum	8%
B. infantis	12%
B. breve	11%

The discrepancies between these findings are probably a result of the differing techniques used to identify the strains; the French and Japanese workers used carbohydrate fermentation, whereas the Italian authors identified species using DNA-DNA hybridization.

CONSTITUENTS OF HUMAN MILK AFFECTING THE EQUILIBRIUM OF THE INFANT'S FLORA. Humanized milks have an organoleptic composition similar to that of human milk: high concentration of lactose, some lactoferrin and lactulose, low concentrations of the proteins used by putrefaction organisms, and a low buffering potential.[141] They are still incapable of providing the conditions favorable to bifidobacteria in breast-fed infants. Human milk provides factors essential to the intestinal development of *Bifidobacterium.*

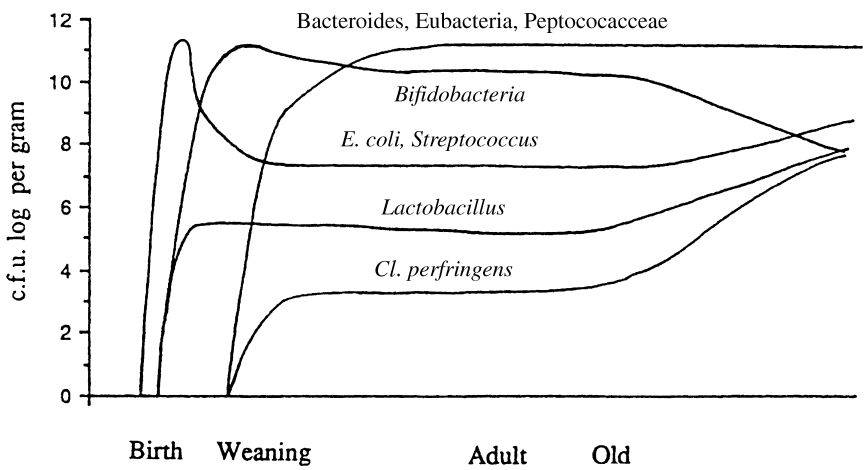

Figure 5 Changes of intestinal flora from birth to old age. (From Mitsuoka, 1984.)

In 1930, Polonowski and Lespagnol[142] isolated oligosaccharides other than lactose from human milk, which they named gynolactose and allolactose [β-D-galactopyranosyl-(1,6)-D-glucopyranose].

Levesque et al.[143] administered relatively large quantities of *N*-acetylglucosamine to infants with low levels of *B. bifidum* in the flora. They then observed the appearance of this species in the stools and its disappearance if the administration was stopped. In children fed milk containing added porcine mucin, the pH of the stools fell and the bifidum population rose.[144]

Numerous substrates therefore appear to be involved in maintaining the equilibrium of the infant's intestinal flora. To date, no active molecule has been identified.

Endogenous Substrates. Endogenous substrates exist within the digestive tract without a dietary source. They are produced by the host and may be used by bacteria. Some strictly anaerobic bacteria produce enzymes able to degrade blood group antigens and mucin oligosaccharides. These bacteria include species of the genus *Bifidobacterium*: *B. bifidum* and *B. infantis*.[145,146] They are able to remove the *N*-acetyl-D-galactosamine residues from the blood group A factors and also secrete α-L-fucosidases, sialidases, and β-glycosidases.[147]

Environment. The country, hospital, and even the unit within which the delivery takes place influence the rapidity of colonization by *Bifidobacterium.* The species present and the biotypes found also vary with time. Mitsuoka et al.[109] isolated mainly *B. infantis* from the stools of Japanese infants, but 10 years later *B. breve* was recognized as the dominant species.

Numerous observations suggest that the environment and, in particular, obstetric and therapeutic customs (increasingly frequent use of antibiotics) play a role in the colonization of neonates by bifidobacteria. It would even appear that very strict conditions of hygiene delay the implantation of *Bifidobacterium*.[138,148,149]

B. Change from Weaning to Adulthood

1. Influence of Age

Many authors observe that the number of bifidobacteria falls significantly in adult stools and particularly in those of the elderly, whereas the numbers of *Bacteroides*, *Eubacterium*, *Peptostreptococcus*, *Clostridium*, Enterobacteriaceae, *Streptococcus*, and *Lactobacillus* increase.[114,150–153] This change is usually due to a reduction in the gastric secretion in this age group. The fall-off in *Bifidobacterium* is accelerated in the elderly, where there is an increase in *Clostridium* and optional aero-anaerobic species. Figure 5 shows the change in the intestinal flora throughout life.

The proportions of the various *Bifidobacterium* species also vary with age, and each age group has its characteristic species (see Table 5). Thus, in children under 7 months of age, Mitsuoka et al.[109] and Biavati [154] isolated *B. infantis*, *B. breve*, *B. longum* biovar. b, and *B. bifidum* var. b. These species, with the exception of *B. longum* biovar. b, are not present in young children and disappear in the flora of older children and adults. *B. infantis* appears to be specific to neonatal infants[35] and to show a higher incidence in the stools of breast-fed infants.

In children and adults, *B. longum* biovar. a, *B. adolescentis*, and species with similar fermentation characteristics (*B. catenulatum*, *B. pseudocatenulatum*) are well

Table 5 Distribution of *Bifidobacterium* Species in Human Colon

Population	Predominating species	Minor species
Breast-fed infants	*B. longum*	
	B. infantis	
	B. breve	
Bottle-fed infants	*B. adolescentis*	*B. bifidum* biovar. b
Children	*B. infantis*	
	B. breve	
	B. bifidum biovar. b	
	B. longum	
Adults	*B. adolescentis* biovars. a and b	*B. bifidum* biovar. a
	B. longum	
Older adults	*B. adolescentis* biovars. b	
	B. longum	

represented. In contrast, *B. infantis* and *B. breve* are not present in this age group,[35,109,140] *B. adolescentis* is the species characteristic of adult flora, and the proportion of *B. adolescentis* type b increases considerably in the elderly.[109,153]

2. Influence of Diet

The high degree of variability of the intestinal flora in infants depending on diet contrasts with the apparent stability of the flora in adults despite differences in diet. It would seem that foodstuffs have little impact on the constitution of the dominant intestinal flora.[123,154] However, Mitsuoka[153] noted a reduction in the level of *Bifidobacterium* after the administration of a western diet to individuals used to Japanese food.

3. Pathogenicity

B. dentium (formerly *Actinomyces eriksonii*) is isolated from dental caries or abscesses, and this species may be confused with four other species, notably *B. adolescentis* (see Section XIII and Table 13).

X. IDENTIFICATION BY PHENOTYPE INVESTIGATION

When the bifidobacteria were discovered by Tissier at the beginning of the twentieth century, taxonomy was based entirely on morphological observations. This lack of differentiation criteria explains the numerous debates which preceded the creation of the genus *Bifidobacterium.* Taxonomy subsequently was based on increasingly numerous phenotype characteristics and today can make use of progress in genotyping.

A. Identification of the Genus *Bifidobacterium*

Until the 1960s, the only identification criteria were phenotype characteristics.

1. Morphology

Since a branched appearance is seen in other bacterial genera (*Arthrobacter*, *Propionibacterium*, *Corynebacterium*, *Actinomyces*), it cannot be considered a specific characteristic but only an indicative criterion.[32]

2. Culture Conditions

Bifidobacteria develop under anaerobic conditions at 37°C in species of human origin or 42°C and higher for species of animal origin and require an incubation time of 48 hours.[21]

3. Metabolites

The determination by gas chromatography of organic acids produced at the end of fermentation and notably an acetic acid/lactic acid ratio of about 3/2 provides excellent identification criteria for the genus *Bifidobacterium.* In addition, it is important to note that bifidobacteria produce the L+ isomer of lactic acid.

4. Enzyme Tests

The association of a branched shape with the presence of fructose-6-phosphate phosphoketolase (F6PPK) in a strain indicates that it belongs to the genus *Bifidobacterium.* The detection of F6PPK can be completed by a test for α-galactosidase. The API ZYM system indicates α-galactosidase activity in bifidobacteria[155] but not in lactobacilli.[156] This test can therefore be used as an identification indicator.[42]

5. Study of Electrophoretic Patterns

All soluble cell protein electrophoretic patterns show a band that migrates over the same distance, with the exception of *B. boum*, for which this band is located at a slightly greater distance from the anode.[157] The presence of this band therefore appears to provide an appreciable criterion for the identification of the genus.

6. Lipids and Constituents of the Cell Wall Membrane

Bifidobacteria have the following fatty acids: $C_{14:0}$ (myristic acid), $C_{16:0}$ (palmitic acid), $C_{16:1}$ (palmitoleic acid), $C_{18:0}$ (stearic acid), and $C_{18:1}$ (oleic acid). In addition, though the genera *Bifidobacterium* and *Lactobacillus* both contain diphosphatidylglycerol and phosphatidylglycerol, only *Bifidobacterium* possesses polyglycerolphospholipids and their lyso derivatives, alanylphosphatidylglycerol, and the lyso derivatives of diphosphatidylglycerol.[158] Analysis of the cell composition in terms of lipids and phospholipids therefore provides a good criterion for distinguishing between the genus *Bifidobacterium* and the Lactobacillaceae. It should be noted that the growth temperature and the composition of the culture medium have a marked influence on the distribution of lipids and phospholipids, although the peptoglycan structure of *Bifidobacterium* is closer to that of the Lactobacillaceae than the Actinomycetaceae.[159,102]

7. Other Identification Criteria

Other tests can be used to identify the genus *Bifidobacterium*, notably:[15,160,161]

Rapid and complete coagulation of milk without the formation of gas
Fermentation of glucose, lactose, levulose, fructose, and galactose, accompanied by marked acidification
No acid production from rhamnose, sorbose, adonitol, dulcitol, erythritol, or glycerol

The development of these microorganisms in peptone water
Negative catalase
No reduction of nitrates
No indole formation
No liquefaction of gelatin
No fermentation of glycerol
No attack of coagulated proteins

B. Species Identification

It is obvious from the multiple taxonomic revisions that have taken place in a few decades how difficult species identification is within the genus *Bifidobacterium.*

1. Sugar Fermentation

This criterion has been used most frequently to identify and define new species. Until 1957, most researchers classed all bifidobacteria together as a single species: *Bifidobacterium difidum.* In 1957, Dehnert[162] was the first to demonstrate the presence of several *Bifidobacterium* biotypes and used 24 sugar fermentation processes to classify the various species into five groups. A few years later, Reuter[163,164] associated serological properties to sugar fermentation to identify new human-derived species isolated from the stools of adults and children and their various biotypes.

Using fermentation profiles and the ability to grow at 46.5°C enabled Mitsuoka[165] to separate human strains from animal strains (pig, chicken, calf, sheep, rat, mouse, guinea pig, bee). He proposed two new species, *B. thermophilum* var. a, b, c, and d, *B. pseusolongum* var. a, b, c, and d, and a new variant, *B. longum* subsp. *animalis* a and b. *B. ruminale* (synonym of *B. thermophilum*) and *B. globosum* and then *B. asteroides*, *B. indicum*, and *B. coryneforme* were isolated in the same year.[166,167]

The ability of a strain to ferment certain sugars is the test first used to identify species. Numerous sugars have been tested, and the results obtained have been compared with the identification tables produced by Mitsuoka[5,153] and Scardovi[20] (Table 6). This method presents no major operating problems but does have several drawbacks: it is lengthy and tedious because a panel of 30 sugars must be studied for 10 days. In addition, the interpretation of the results using identification tables remains controversial and can at best give an indication of an identification based on the fundamental characteristics not open to doubt, for example:

B. longum ferments melezitose, whereas *B. animalis* is unable to ferment this sugar.
B. pseudolongum ferments pentoses and starch, whereas *B. thermophilum* does not ferment pentoses but does ferment starch.
B. breve ferments ribose, mannitol, esculine, and amygdaline but does not ferment arabinose or xylose.
B. infantis does not ferment arabinose, whereas *B. longum* ferments arabinose and melezitose.

Roy et al.[155] developed a rapid method for identifying bifidobacteria species based on the fermentation of seven sugars: arabinose, cellobiose, lactose, mannose, melezitose, ribose, and salicin. A mixture of these seven sugars is monitored by gas chromatography and should make it possible to identify six to eight typical strains of *Bifidobacterium* in less than 24 hours.

Table 6 Sugar Fermentation by *Bifidobacterium* sp.

	D-Ribose	L-Arabinose	Lactate	Cellobiose	Melezitose	Raffinose	Sorbitol	Starch	Gluconate	Xylose	Mannose	Fructose	Galactose	Sucrose	Maltose	Trehalose	Melibiose	Mannitol	Inulin	Salicin
B. bifidum	−	−	+	−	−	−	−	−	−	−	−	+	+	v	−	−	v	−	−	−
B. longum	+	+	+	−	+	+	−	−	−	v	v	+	+	+	+	−	+	−	−	−
B. infantis	+	−	+	−	−	+	−	−	−	v	v	+	+	+	+	−	+	−	v	−
B. breve	+	−	+	v	v	+	v	−	−	−	+	+	+	+	+	v	+	v	v	+
B. adolescentis	+	+	+	+	+	+	v	+	+	+	v	+	+	+	+	v	+	v	v	+
B. angulatum	+	+	+	−	−	+	v	+	−	+	+	+	+	+	+	−	+	−	+	+
B. catenulatum	+	+	+	+	−	+	+	−	v	+	−	+	+	+	+	v	+	v	v	+
B. pseudocatenulatum	+	+	+	v	−	+	v	+	v	+	+	+	+	+	+	v	+	−	−	+
B. dentium	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+
B. globosum	+	v	+	−	−	+	−	+	−	v	−	+	+	+	+	−	+	−	−	−
B. pseudolongum	+	+	v	v	v	+	−	+	−	+	+	+	+	+	+	−	+	−	−	−
B. cuniculi	−	+	−	−	−	−	−	+	−		−	−	+	+	+	−	+	−	−	−
B. choerinum	−	−	+	−	−	+	−	+	−	−	−	−	+	+	+	−	+	−	−	−
B. animalis	+	+	+	v	v	+	−	+	−	+	v	+	+	+	+	v	+	−	−	+
B. thermophilum	−	−	v	v	v	+	−	+	−	−	−	+	+	+	+	v	+	−	v	v
B. boum	−	−	v	−	−	+	−	+	−	+	−	−	−	+	+	−	+	−	+	−
B. magnum	+	+	+	−	−	+	−	−	−	+	−	+	+	+	+		+	−	−	−
B. pullorum	+	+	−	−	−	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+
B. suis	−	+	+	−	−	+	−	−	−	+	v	v	+	+	+	−	+	−	−	−
B. minimum	−	−	−	−	−	−	−	+	−	−	−	+	−	+	+	−	−	−	−	−
B. subtile	+	−	−	−	+	+	+	+	+	−	−	+	+	+	+	v	+	−	v	v
B. coryneforme	+	+	−	+	−	+	−	−	+	+	−	+		+	+	−	+	−		+
B. asteroides	+	+	−	+	−	+	−	−	v	+	−	+	v	+	v	−	+	−	−	+
B. indicum	+	−	−	+	−	+	−	−	+	−	v	+	v	+	v	−	+	−	−	+

+ = positive; − = negative; v = variable.

2. Study of F6PPK Isoenzymes

A test using a colorimetric reaction following starch gel electrophoresis for three isoenzymes of F6PPK can give an indication of species identity.[35] These isoenzymes catalyze the same reaction but are distinguished by differing electrophoretic patterns. The migration distances are linked to the ecological origin of the species: human (15 cm), mammalian (10 cm), or bee[20] (Table 7). In addition, purified preparations of F6PPK from *B. globosum* (mammalian origin) and *B. dentium* (human origin) demonstrate activities which vary with regard to optimum pH, the identity of the metal inducing maximum activity, heat inactivation, molecular weight, and affinity toward the substrate.[104]

3. Study of Protein Profiles

A bacterial strain cultured under standard conditions always gives the same protein profiles. The sequence of amino acids, the molecular weight, and the net electrical charge of each protein are determined by the sequence of nucleotides in the DNA. The protein profile of each strain is therefore a fingerprint of the genome. The cell proteins are dissolved using detergents such as SDS, but many studies have been carried out using only the soluble fraction of disintegrated cells.[16] Two types of study can be envisaged for the comparison of *Bifidobacterium* species with each other.

Electrophoresis in a starch gel (zymogram) of the 14 enzymes of transaldolase and the 19 isoenzyme of 6-phosphogluconate dehydrogenase (6PGD) can be used to compare the electrophoretic mobility of these enzymes in the original strain (Table 8). A colorimetric method applied to 3-phosphoglyceraldehyde dehydrogenase is able to identify other strains.[168] The electrophoretic migration distances for F6PPK appear to be linked to the ecological origin of the species, but the same is not true for the other glucose metabolism enzymes of *Bifidobacterium*—transaldolase, transketolase, 6-phosphogluconate dehydrogenase, and aldolase.[169]

Electrophoresis in a polyacrylamide gel of the lysate of a strain provides electrophoretic profiles of the soluble cell proteins. The distribution of the protein bands is

Table 7 Migration of F6PPK in *Bifidobacterium* sp.

Species	Migration (cm)	Species	Migration (cm)
B. bifidum	15	*B. choerinum*	n.d.
B. longum	15	*B. animalis*	10
B. infantis	15	*B. thermophilum*	10
B. breve	15	*B. boum*	n.d.
B. adolescentis	15	*B. magnum*	10
B. angulatum	15	*B. pullorum*	10
B. catenulatum	15	*B. suis*	10
B. pseudocatenulatum	n.d.	*B. minimum*	10
B. dentium	15	*B. subtile*	10–15
B. globosum	10	*B. coryneforme*	16
B. pseudolongum	10	*B. asteroides*	16
B. cuniculi	n.d.	*B. indicum*	16

Note: n.d., not determined.
Source: Scardovi, 1986.

Table 8 Migration of Transaldolase and 6PGD in *Bifidobacterium* sp.

Species	Electrophoretic pattern	
	Transaldolase	6PGD
B. bifidum	7	7-(8)
B. longum	(5)-6-8[a]	5-(6[a])
B. infantis	5-(6)-(8[a])	(3)-4[a]-(5)
B. breve	6	(5)-6-7
B. adolescentis	8	5
B. angulantum	5	5
B. catenulatum	5	6[a]-8
B. pseudocatenulatum	4[a]-(5)	1[a]-3
B. dentium	4	(2)
B. globosum	2	(3)-(4)-(5)-6-(7)
B. pseudolongum	2	7
B. cuniculi	1	4
B. choerinum	3	4
B. animalis	5	8-9[a]
B. thermophilum	(7)-8[a]	7-8-9[a]
B. boum	6	8[a]-9
B. magnum	5	7
B. pullorum	2	Absent
B. suis	6	5-8
B. minimum	10	6
B. subtile	3	2
B. coryneforme	6	6
B. asteroides	(6)-(7)-8-(9)	(9)-10[a]-(11)-(12)-(13)
B. indicum	(6)-7-8-9[a]	6-(7)-8-(9[a])

[a]Number of isoenzymes for type strains.
( )Number of isoenzymes in less than 10% of strains.
Source: Scardovi, 1986.

then compared with those for a reference strain.[35,157,170] This method is doubtless the most discriminating and is both reliable and sensitive, since it is able to distinguish between strains with DNA-DNA homology levels of up to 80%,[157] but it is an onerous method, requiring reference strains, and is difficult to interpret.

Use of these two types of electrophoresis has given the following results:

1. The homology between *B. dentium* (the only species thought to be pathogenic) and *B. eriksonii* (formerly *Actinomyces eriksonii*) was established by comparing their electrophoretic patterns (zymograms).[169] This identity complies with high DNA-DNA homology (80–100%).
2. Electrophoresis in polyacrylamide gel enabled Biavati et al.[157] to recognize four new species: *B. minimum*, *B. subtile*, *B. cornyeforme*, and *B. globosum*, which is now distinguished from *B. pseudolongum.*
3. *B. adolescentis* and *B. dentium*, which have identical phenotype profiles, can also be differentiated by their zymograms (starch gel electrophoresis), which differ.[169] Polyacrylamide gel electrophoresis also confirms these findings.[157]

4. The comparison of zymograms and protein electrophoretic patterns in polyacrylamide gel of *B. infantis* and *B. longum* is interesting. These two species have the same isoenzymes of transaldolases, that is, three different isoenzymes, which migrate to a distance of 5, 6, or 8 units. The only difference is the incidence within the strain: the isoenzyme migrating to a distance of 5 occurs more frequently in *B. infantis*, and that which goes furthest, to 8, is found most frequently in *B. longum*.[169] The bands obtained on the electrophoretic diagrams of these two species show an identical distribution. Only the concentrations of the proteins differ.[35,157]

We are therefore faced by a very unusual phenomenon: these strains, although they belong to different species, present similar profiles, thus defining a "continuum." Table 8 shows that the transaldolases and 6PGD of *B. adolescentis* with electrophoretic mobilities of 8 and 5, respectively, are found in 50% of *B. longum* and in many strains of *B. infantis*, highlighting what is doubtless a very close degree of relatedness between these species. In contrast, the electrophoresis patterns of the cell proteins for these three strains differ, confirming that they are indeed three separate species.[36,157,171]

The zymogram of *B. bifidum* is in contrast highly specific. This species is the only one to show a transaldolase and F6PPK migration distance of 7 units. Only a few strains of *B. thermophilum* of bovine origin are similar. In this case, the differentiation is based on the mobility of the 3-phosphoglyceraldehyde dehydrogenase.

Study of the protein patterns provides valuable information about a given strain, and the numerical analysis of the patterns of a large number of strains makes it possible to achieve:

Rapid grouping of the strains.
Archiving of a large number of models in a reference data bank.
The attribution of unknown bacteria to their group and their possible identification.
A quick method of determining whether two colony types in a culture are due to variation or contamination.
Determining the homogeneity or heterogeneity of the taxa.

When the preparation conditions for the extracts and their electrophoresis are standardized, a high degree of reproducibility (in excess of 96%) can be obtained.[172]

4. Transaldolase Serology

An immunological approach investigating the serology of the transaldolases can also be used to differentiate between species within the genus *Bifidobacterium.* Figure 6 shows the results of the studies performed.[107,173,174]

This method consists of preparing immunosera against the highly purified transaldolases of eight species of *Bifidobacterium*, *B. infantis*, *B. angulatum*, *B. globosum*, *B. thermophilum*, *B. suis*, *B. cuniculi*, *B. minimum*, and *B. asteroides* and testing them against 21 bacterial species of the genus in order to determine their immunological distances. These results, expressed as taxonomic distance, are shown in the dendogram (Fig. 6). This diagram illustrates the interrelationships existing within the genus and shows that the seven groups defined by Sgorbati and London[107] (A, B, C, D, E, F, and G) detected by this model can be split into four distinct groups closely linked at the ecological origin of the species. These four antigenic groups (I, II, III, and IV) coincide with the arrangement

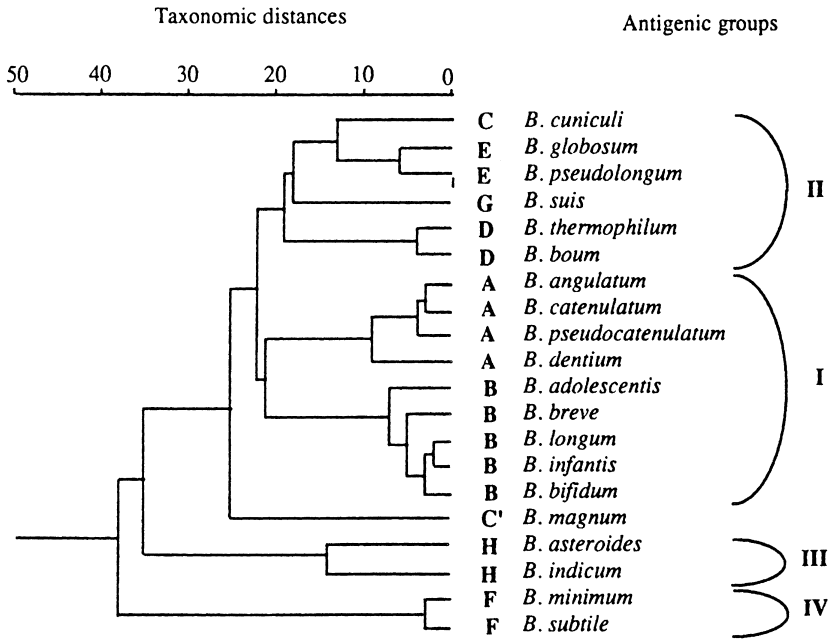

Figure 6 Immunological relationships among transaldolases in *Bifidobacterium.* (See text for details.)

of the species of *Bifidobacterium* based on electrophoretic mobility[36,174,175] and are also confirmed by DNA-DNA hybridization studies.

In addition, this dendogram is able to distinguish two subgroups, A and B, among the strains of human origin (I): (1) *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum*, and *B. dentium*; and (2) *B. adolescentis*, *B. breve*, *B. longum*, *B. infantis*, and *B. bifidum*.[174] The species associated with mammalian animal habitats (II) belong to groups C, D, E, and G. The two species found only in the bee (group H) are antigenetically very distant from the other members of the genus (III). The most widely separated species (IV) (group F) were isolated from waste water.

5. Enzymes

B. breve is one of the few species to produce β-glucuronidase activity.[41] It is suspected that this enzyme may convert procarcinogens into carcinogens.[176] In addition, *B. longum* is the only species to have neither β-glucosidase nor *N*-acetylglucosaminidase activity.

6. Composition of the Wall

The sugar composition of the wall varies with strain, particularly with regard to the percentage of rhamnose and glucose[15] (Table 9). The sequence of amino acids in the peptidoglycan may vary from one species to another, making it possible to separate species from which are relatively close to each other, such as *B. boum* from *B. thermophilum* or *B. minimum* from *B. subtile*.[102] In addition, Bezirtzoglou[110] noted that only the species *B. bifidum* has a poly-(1,2)-glycerophosphate skeleton in the lipoteichoic acids, which is substituted in the end position by a polysaccharide.

Table 9 Cell Wall Composition of *Bifidobacterium* sp.

Species	Origin	Kind of peptidoglycan cross linkage	Polysaccharide		
			Glucose	Galactose	Rhamnose
B. bifidum	Adult stool	Orn-Ser-Asp-Ala	+	+	+
B. infantis	Infant stool	Lys-Gly	+	+	+
B. breve	Infant stool	Lys-Gly	+	+	+
B. liberorum	Infant stool	Lys-Gly	+	+	+
B. parvulorum	Infant stool	Lys-Gly	+	+	+
B. asteroides	Bee	Lys-Gly	+	+	−
B. suis	Pig	Orn or (Lys)-Ser-Ala-Thr-Ala	+	+	(+)
B. longum	Adult stool	Orn or (Lys)-Ser-Ala-Thr-Ala	+	+	+
B. thermophilum	Pig	Orn-(Lys)-Glu	+	+	+
B. adolescentis	Adult stool	Lys or (Orn)-Asp	+	+	−
B. indicum	Bee	Lys-Asp	−	+	+
B. pseudolongum	Pig	Orn or (Lys)-Ala	+	+	+

Op Den Camp et al.[177] prepared antibodies to the lipoteichoic acids of *B. bifidum* by coupling with an immunogenic protein. They were specific towards the polyglycerol phosphate core (essentially poly 1,2) and to a small extent to the polysaccharide portion.

Crossed reaction tests with phenolic extracts of lipoteichoic acids of *Bifidobacterium* and *Lactobacillus* have shown that only the former react, making it possible to envisage a serogroup with lipoteichoic acids as group antigens.

7. Processing of the Results

All these identification criteria give responses that must be classified and interpreted. Most of the problems involved in processing data tables resulting from the examination of the physiological and biochemical properties of the bacteria are solved by the use of computer-assisted numerical taxonomy. The usefulness of numerical taxonomy depends on several factors:[167]

1. Strain selection
2. Number of characteristics examined (greater than 50)
3. Rigorous standardization of the methods of analysis
4. Weight attributed to each characteristic in the evaluation
5. Classification of the reactions as positive, negative, or noncomparable
6. Type of computer software used

XI. IDENTIFICATION BY STUDY OF THE GENOME

A. Identification of the Genus *Bifidobacterium*

The DNA base composition of the chromosome of *Bifidobacterium* differs from that of *Lactobacillus* [17] and other lactic bacteria.

Genus	GC%
Lactobacillus	34.7–50.8
Streptococcus	33–44
Leuconostoc	39–42
Bifidobacterium	57.2–64.5

However, two organisms with similar G + C (GC%) analysis are not necessarily closely related. The GC% cannot therefore be considered as an exclusion characteristic in bacterial taxonomy.

B. Species Identification

1. DNA Base Composition

B. longum is distinguished from *B. animalis* and *B. pseudolongum* by its GC%. The GC% of *B. longum* is 58, whereas that of *B. animalis* and *B. pseudolongum* is 60.

2. DNA-DNA Hybridization

DNA-DNA homology is a mean measurement of similarity in which the entire genome of one organism is compared with that of another. A fragment of denatured DNA from a reference strain is labeled and then used as a probe to hybridize with a single strand of DNA from the strain to be identified. The more DNA base pairing there is between two strains, the closer they are genetically.

The use of DNA-DNA hybridization methods has advanced the taxonomy of the bifidobacteria.[175,178] On the basis of the DNA-DNA homology percentages, 11 different species can be described: *B. indicum*, *B. coryneform*, *B. asteroides*, *B. ruminale*, *B. globosum*, *B. suis*, *B. pullorum*, *B. magnum*, *B. catenulatum*, *B. dentium*, and *B. angulatum*.[167,179–182] The fact that *B. bifidum* and *B. longum* belonged to two different species has been confirmed, but the following species have been combined to form a single species:

B. lactentis, *B. liberorum*, and *B. infantis* under the name of *B. infantis*
B. ruminale and *B. thermophilum*
B. breve and *B. parvulorum* under the name of *B. breve*
B. pseudolongum and *B. globosum* [175]

In the following year and using the same methods, Holdeman and Moore[183] divided *B. infantis* into three subspecies and *B. adolescentis* into four groups. In addition, they described two new species: *B. cornutum* and *B. eriksonii*.

In 1974, Scardovi and Trovatelli[184] detected genetic differences between *B. longum* subsp. *longum* and *B. longum* subsp. *animalis* and proposed raising these subspecies to rank of species: *B. animalis*, *B. longum*, and *B. animalis*.

A few years later, some new species were described by Scardovi[21]—*B. pseudocatenulatum*, *B. cuniculi*, *B. choerinum*, and *B. boum*—and then, in 1982, by Biavati et al.[157]—*B. minimum* and *B. subtile*.

The results of DNA-DNA hybridizations between the various species of *Bifidobacterium* are shown in Table 10. From this table it can be seen that two continua can be defined from the human-defined strains *B. infantis* and *B. longum* (50–76%

Table 10 DNA-DNA Homology in the Genus *Bifidobacterium*

	B. bifidum	*B. longum*	*B. infantis*	*B. breve*	*B. adolescentis*	*B. angulatum*	*B. catenulatum*	*B. pseudocatenulatum*	*B. dentium*	*B. globosum*	*B. pseudolongum*	*B. cuniculi*	*B. choerinum*	*B. animalis*	*B. thermophilum*	*B. boum*	*B. magnum*	*B. pullorum*	*B. suis*	*B. minimum*	*B. subtile*	*B. asteroides*	*B. indicum*
B. bifidum	100	*	*	*	0		*	*		0		*	*	*	*	*	*	*	0	*	*	0	0
B. longum	/	88	63	*	0		*	*		0		*	*	*	0	*	*	*	0	*	*	0	
B. infantis	/	65	88	*			*	*		0		*	*	*	*	*	*	*	0	*	*	0	
B. breve	*	/	/	100			*	*		0		*	*	*	*	*	*	*	0	*	*	0	
B. adolescentis	*		*	*	86	*	*	*	*			*	*	*		*	*	*		*	*		
B. angulantum	*	*	*	*	*	88	*	*	*	*		*	*	*	*	*	*	0	*	*	*		
B. catenulatum	*	*	*	*	/	*	90	65	*	*		*	*	*	*	*	*	*	*	*	*		
B. pseudocatenulatum							67	97				*	*			*							
B. dentium	*	*	*	*	*	*	*	*	90	*		*	*	*	*	0	*	0	*	*	0		
B. globosum	*		*	*				*		100	75	/	/	/	*	*	*	*	*	*	*	*	*
B. pseudolongum	*		*	*				*		67	100	*	/			*			*				
B. cuniculi	*		*	*				*		/	*	98	*	*	*	*		*					
B. choerinum	*		*	*				*		/	/	*	98	*	*	*		*					
B. animalis	*	*	*	*	*	*	*	*		*	*	*	*	86	*	*	*	0	*	*	*	*	0
B. thermophilum	*		*	*				*		*	*	*	*	*	98	/	*	*	*	*	*	0	0
B. boum	*		*	*				*				*	*	*	/	82		0					
B. magnum	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	90	*	*	*	*		
B. pullorum	*		*	*				*				*	*	*		*		102					
B. suis	*		*	*				*		*	0	*	*	*	*	*	*	*	100	*	*		
B. minimum	*	*	*	*	*	*	*	*	0	*		*	*	*	*	0	*	*	*	102	*	*	0
B. subtile	*	*	*	*	*	*	*	*	0	*		*	*	*	*	0	0	*	*	*	85	*	*
B. coryneforme				*						*		*	*	0				0		*	0	*	/
B. asteroides				*				*		0		*	/		0	0		*	0	0	*	100	*
B. indicum				*						0		*	*		10			*		0	0	*	100

*Value between 40% and 60%.

homology), which had already been detected by the zymogram, electrophoresis of soluble proteins, and growth factor requirements[57] and suspected by Scardovi et al.[175] The other continuum is that of *B. catenulatum* and *B. pseudocatenulatum* (60–80% homology).[174]

These genotypic findings nonetheless raise some questions:

1. The electrophoretic mobility of the transaldolases and 6PGD had suggested proximity of *B. adolescentis*, *B. longum*, and *B. infantis*. However, although the DNA-DNA hybridizations between these strains confirm the relationship between *B. longum* and *B. infantis*, *B. adolescentis*, on the contrary, appears to be genetically very distant from *B. longum* (0% hybridization).
2. The serology of the transaldolases makes it possible to separate the strains of human origin into two subgroups, and *B. adolescentis* appears to belong to the subgroup as *B. breve*, *B. longum*, *B. infantis*, and *B. bifidum* (and not to the subgroup containing *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum*, and *B. dentium*). The hybridizations challenge this finding since, genetically, *B. adolescentis* appears to be distant from these four species (less than 20% homology) but close to the species in the other subgroup, in particular to *B. dentium* (some strains showing up to 49–57% homology). In contrast, the results obtained by electrophoresis of soluble proteins and the zymograms appear to distance *B. adolescentis* and *B. dentium*.

3. Plasmid Tests

The number of plasmids detected in a strain of *Bifidobacterium* may suggest the identification of certain species because only some possess plasmids.[104,105] This technique is particularly useful for the separation of *B. longum* and *B. infantis*.

4. New Methods

Today, hope is based on the use of new methods, which should lead to further changes.

The whole bacterial DNA can be processed using restriction enzymes.[185] But even if the genome is relatively small, there are too many restriction fragments for the electrophoretic pattern to be easily read. This method can demonstrate that two strains are different, but can in no case be used to confirm the similarity of two bacteria.[186] The successive use of several restriction enzymes should make it possible to pinpoint identification.

Grimont and Grimont[187] suggest that the parameters used to describe species and strains should include the size of the DNAr restriction fragments following agar medium electrophoresis. The DNA fragments carrying genes (DNAr) coding for ribosomal RNA are then localized on filters by hybridizing with either a DNAr $16 + 23$S from *Escherichia coli* or with a DNA probe that codes for the well-conserved portions of ribosomal RNA 5S or 16S.

Many more studies are required before a new level of identification can be defined that could be achieved by sequencing the RNAr 5S and 16S, which are very well-conserved molecules throughout the animal kingdom. Comparison of these sequences with those of reference strains should give information extremely useful for identification.[187]

DNA-DNAr hybridization remains to be tested regarding its value in improving the classification of bifidobacteria.

Pulsed field electrophoresis requires large fragments of DNA. Studies have been carried out in order to identify restriction enzymes able to cut DNA at infrequent restriction sites.

C. Conclusion

The identification of species within the genus *Bifidobacterium* is difficult. The kits and commercial tests that provide phenotype identification information are not appropriate for these bacteria, and their use does not provide a sufficiently reliable answer. Even with 100 phenotype characters, the identification is not definite.

Genotyping today provides a more accurate and reliable approach to bacterial taxonomy. DNA-DNA hybridization is widely used and is a particularly appropriate method since it makes it possible to study the entire bacterial genome, including those parts that do not code for proteins. The new genome methods now under investigation hold the promise of a more accurate and quicker identification method.

Table 11 shows the accuracy and precision of identification obtained using genotyping. Several species that cannot be differentiated by phenotyping can be distinguished by their genetic characteristics. DNA-DNA hybridization methods take into account the entire genome, whereas phenotype analysis involves only the segments of the genome in which the phenotype expression can be measured[188]—about 10%.

Table 11 Relationship Between DNA-DNA Homology and Phenotypy

Genotype	Phenotype
B. minimum	*B. minimum*
B. bifidum	*B. bifidum* a and b
B. thermophilum	
B. boum	*B. thermophilum*
B. choerinum	
B. subtile	
B. pseudolongum	*B. pseudolongum*
B. globosum	
B. animalis	
B. magnum	*B. animalis*
B. suis	
B. pullorum	
B. cuniculi	*B. cuniculi*
B. longum	*B. longum* a and b
	B. infantis ss. *infantis* a and b
B. infantis	*B. infantis* ss. *liberorum*
	B. infantis ss. *lactentis*
B. breve	*B. breve* ss. *breve* a and b
	B. breve ss. *parvulorum* a and b
B. adolescentis	
B. dentium	
B. catenulatum	*B. adolescentis* a, b, c, and d
B. pseudocatenulatum	
B. angulatum	
B. asteroides	*B. asteroides*
B. coryneforme	
B. indicum	*B. indicum*
B. gallinarum	*B. gallinarum*

Note: DNA-DNA homology higher than 50%.

XII. RELATIONSHIP BETWEEN INTESTINAL FLORA AND INTESTINAL CELLS: ADHESION AND COLONIZATION METHODS

The study of the mechanisms of adhesion has been facilitated by the development of investigation methods and can be carried out today in vivo by taking a human or animal colon biopsy sample, which is then examined under electron microscopy or in vivo by the culture of intestinal cells or tissues under survival conditions and exposed to bacteria before being examined under the electron microscope.

A. Colonization Conditions

To obtain a probiotic or pathogenic effect depending on the invasive bacterial species concerned, the bacteria must adhere to the cell surfaces of the digestive tract. Two conditions must be present to allow the implantation of a bacterium:

1. It must be able to multiply, depending on the presence of a substrate and a redox potential appropriate for its growth requirements.
2. It must be able to reside in situ, i.e., adhere to the cells or mucus and avoid expulsion.[189]

B. Mechanism of Adhesion

There are numerous conflicting hypotheses concerning the possible implantation of ingested microorganisms. These bacteria may either simply pass through or colonize the intestine by adhering to the cell wall, in which case they belong to the subdominant flora.

In most cases, there is a uniform interstice between the bacterium and the host cell measuring less than 40 nm, which is filled with fibrillar material.[190] The bacterium may be completely encircled by the membrane of the apical pole of the cell or only partially surrounded, and in some cases the association takes place without penetration. This adhesiveness depends on three factors: adhesins, adhesin receptors, and mucus.

1. The Adhesins

These structures, which are implied in the attachment process and are present in some bacteria, may be of two types.

Protein Type. These are either external membrane proteins or individualized structures such as the pili or fimbriae of gram-negative bacteria.[191] The pili may be bound to the glycoproteins and glycolipids of the cell membrane of many vertebrate species.[189,190] These non-protein structures may also be fibrillar structures other than fimbriae. They have been described in the superficial adhesion process of enteropathogenic *E. coli*.[192]

Elements known as "fimbriosomes" have also been detected in hyperadhesive mutant strains. Fimbriosomes are rounded structures closely associated with the fimbriae.[193] It has not yet been demonstrated whether these structures potentiate the effects of the fimbriae or whether they act separately.

Nonprotein Type. These are the polysaccharides of the capsule or slime. The polysaccharide fraction of *B. infantis* is involved in the adhesion of this bacterium to

the epithelial cells of the ileum or the lipotechoic acids (LTAs) of gram-positive bacteria.[194,195]

Many studies have demonstrated that the LTA of gram-positive bacteria have a high binding affinity to the membranes of the epithelial cells of mammals.[196–199] Binding occurs spontaneously through the intermediary of the lipid fraction of the LTAs.[199] The binding of the LIAs of the bifidobacteria to human epithelial cells in culture is dependent on cell concentration and time and appears to be reversible. LTA is bound through the fatty acids, which are themselves bound to the esters.[177]

An important phenomenon that should be highlighted is "phase variation," which allows a bacterium to modify its surface and thus its adhesive potential, depending on its phase. This phenomenon is familiar in *E. coli* both in vitro and in vivo.[190]

2. Adhesin Receptors

The most probable receptor on the membrane of the intestinal epithelial cells is a protein or glycoprotein with fatty acid–binding sites.[177] However, the adhesion of *E. coli* to uroepithelial cells and isolated human colon cells appears to involve the intermediary of glycolipids (glycolipids account for about 20% of the membrane lipids of the enterocyte) containing Galα 1–4 Galβ residues, as well as through the intermediary of receptors which are sensitive to mannose and which could consist of one or several glycoproteins.[200] It appears that the Gal α 1–4 Gal β receptors are indeed irregularly distributed among intestinal cells.[201] The surface receptors trap the bacteria in mucus secretions.

The membrane receptivity of the host cells is strongly influenced by cell maturity,[202] the age of the host[193,203] and the portion of the digestive tract involved. This results from differences of structure and composition of the enterocytes of the host, which are mature in adults and immature in young subjects.[204] The differences found in the composition of the intestinal flora in infants, children, and adults may be related to this change of receptors in function of age. The type and quantity of specific glycolipid receptors is also determined genetically.[205]

3. Mucus

Mucus covers the entire mucosa of the digestive tract from the stomach to the colon, and it consists of a sort of protective elastic and viscous gel consisting of glycoproteins. Its most obvious function is to provide specific protection against bacterial penetration, but its presence is also essential in the mechanisms of bacterial adhesion. Using electron microscopy, Croucher et al.[206] have confirmed the close association of bacteria with the mucus layer.

C. Analysis of the Adherent Flora

Compared to the diversity of the intraluminal flora, the flora adhering to the epithelium is generally limited to a few species of bacteria. The bacterial concentrations are generally lower by one or two log factors than the intraluminal populations. If biopsies are taken from various points of the duodenum and jejunum, the bacterial population appears to be equally distributed along a given segment of the gastrointestinal tract.[207] Despite the marked predominance of anaerobic bacteria in the lower portion of the gastrointestinal tract, their relationship with the epithelium of the colon or rectum has not been studied. Only the adhesive capacities of the optional anaerobes (such as enterobacteria) have

been studied, and few studies have been devoted to this topic. The adhesion of *E. coli* and other enterobacteria has been demonstrated by electronmicroscopy of biopsies.[208,209] The density of bacteria ranges from 10^6 to 10^7 cfu/g of fresh tissue.

Examination of colonic biopsies allowed Hartley et al.[208] to demonstrate that the same strain of *E. coli* is closely associated with the intestinal wall throughout the length of the colon.

D. Adhesion of the Bifidobacteria

Sato et al.[194] have demonstrated the role played by the polysaccharides in the adhesion of the bifidobacteria by inhibiting this phenomenon using antibodies targeted against polysaccharides. Gram staining is negative in cell-bound *Bifidobacterium*. This phenomenon reflects an increase in the membrane permeability of these bacteria. The bonds that permit this adhesion are probably strong.

The characteristics of the epithelial cell of the human colon/lipoteichoic acids of *Bifidobacterium* complex have been investigated by determining the radioactivity of carbon-14–labeled LTAs after interaction with suspended colonocytes. The most probable receptor on the colonocyte membrane is a protein or glycoprotein with fatty acid–binding sites.[210]

The adhesion of *Bifidobacterium* and more particularly the hydrophobic interactions are promoted by a high level of fatty acids in the LTAs, resu1ting in a high level of hydrophobicity of the bacterium. The strong electrostatic charge of the polysaccharides of gram-positive bacteria also favors adhesion.[189]

E. Modifications of Adhesion

Several studies have shown that some bacterial species may modify the receptor sites of epithelial cells, resulting in an inhibition of the adhesion of the microorganisms which use them. Thus, extracellular enzymes of *Bifidobacterium* may degrade specific sites of pathogenic organisms or their toxins.[189,190,211] These enzymes, some of which are glycosidases, may degrade the receptors within the cell or mucus and also eliminate any bacteria bound to them.[202]

XIII. THE INTESTINAL FLORA

Knowledge of the intestinal flora has developed simultaneously with the methods of investigation with regard to both sampling technique and analysis of the flora.

A. Methods of Evaluation

Conventional stool collection allows only investigation of the terminal flora of the digestive tract. The dominant flora consists of highly anaerobic microorganisms, which are therefore difficult to isolate and keep alive. More elaborate sampling methods, such as biopsy of the intestinal mucosa and collection of luminal aspirate fluid using a Camus probe or weighted tube, have made it possible to carry out detailed exploration of all portions of the digestive tract. These new sampling methods and the design of more appropriate culture media for the various species now make it possible to use a good direct

approach to understanding the intestinal bacterial flora by identifying and counting the species samples.

The importance and role of the microbial flora within the host can also be assessed indirectly by determining the bacterial metabolites produced using the following methods:

The breath test or measurement of the hydrogen expired by a subject after ingesting lactulose.

Measurement of the activities of various bacterial enzymes in intestinal samples.

Tests for fatty acids excreted in the stools by gas chromatography reflecting bacterial metabolism.

Counting of a given species of *Bacteroides* from a mixture of bacterial DNA isolated directly from the stools and exposed to a species-specific labeled DNA probe has been suggested.[212,213] However, this method would not make it possible to differentiate between viable and nonviable bacteria, and a confirmation of the percentage of live bacteria would still have to be carried out using conventional dish culture methods.

B. Composition

The intestinal flora of a human weighs between 1 and 2 kg, that is, roughly the same weight as organs such as liver, brain, or lungs. The digestive tract houses about 10^{14} bacteria, which means there are more living entities in the flora than there are cells in a normal body and the bacteria consist of 500 species. This flora can be divided into two categories.

1. The dominant population, the effects of which on the host were the first to be understood.
2. The subdominant population, which accounts for less than 1% of the total bacterial population but which, according to studies,[146] may play a nonnegligible role in the equilibrium of the intestinal ecosystem.

Numerous studies have been carried out to define the composition of the intestinal flora. The main results, shown in Table 12, show some discrepancies, which can easily be explained from the choice of culture methods, isolation media, and counting methods. Generally, the most numerous population in adults is *Bacteroides* (about $10^{10.3}$/g of feces). Thereafter, in decreasing order, come *Eubacterium*, *Bifidobacterium*, and then the Peptococcaceae. Of the aero-anaerobes, there are the enterobacteria ($10^{8.2}$) followed by *Streptococcus*, aerobic *Lactobacillus*, and finally *Staphylococcus* ($10^{4.4}$).[5]

C. Factors Affecting the Flora

1. Factors Ensuring the Equilibrium of the Intestinal Flora

The diversity of bacterial species and their quantity at various levels within the digestive tract can be preserved only by means of physical, chemical, and biological regulatory mechanisms.

Intestinal peristaltism results in the elimination of many microorganisms.

The acidity of the stomach maintains a low concentration of bacteria in the upper part of the digestive tract and destroys some pathogens.

The interactions that exist between various bacterial species are also important in maintaining the equilibrium of the intestinal microflora. It is possible to observe

Table 12 Fecal Flora of Different Human Groups

Bacterian group	Infant, 1–4 days	Infant, 5–90 days	Infant, 4–6 years	Adult, 20–64 years	Adult, 65–86 years
Total bacteria[a]	10.1	10.5	10.8	10.8	10.5
Aerobic or facultative anaerobic					
Enterobacteria	9.3	8.8	8.0	8.2	7.8
Streptococcus	8.5	8.1	7.8	7.7	8.2
Lactobacillus	6.4	7.3	7.0	6.7	8.0
Staphylococcus	6.2	6.8	4.0	4.4	4.3
Yeast	3.5	4.0	4.2	3.7	4.6
Anaerobic					
Bacteroides	8.6	8.2	10.4	10.3	10.0
Eubacteria	0	9.7	9.9	9.9	9.5
Bifidobacterium	9.3	9.9	10.1	9.8	9.4
Peptococcus	0	9.0	8.2	8.9	7.7
Clostridium perfringens	5.9	6.9	5.7	4.8	6.6
Veillonella	5.6	6.3	5.2	4.8	6.1

[a] log cfu.
Source: Ref. [153].

symbioses between species as a result of the production of vitamins or amino acids or other metabolites which can be assimilated by other species and also of antagonisms due to the release of antibiotics, bacteriocins, or factors such as the volatile fatty acids.

2. Location and Physiology

The composition of the intestinal flora varies depending on the rate of transit and luminal secretions as well as the intestinal segment.[214] Thus, a given well-defined resident flora corresponds to each portion of the tract. The various factors active along the digestive tract result in qualitative and quantitative differences in the digestive flora, as shown in Table 13. Thus, the flora present in the proximal small intestine (duodenum and jejunum) consists of aerobic gram-positive microorganisms (streptococci and staphylococci) and a few yeasts.

This aero-anaerobic flora is subsequently replaced, within the ileum, by a flora consisting of *E. coli* and anaerobes such as *Clostridium*, *Fusobacterium*, and *Bacteroides* (10^6 total bacteria per mL).[5] This switch from a dominant aerobic population within the stomach to a strictly anaerobic population within the colon can be explained if we accept that the aero-anaerobic bacteria use any oxygen present, creating the redox conditions for the implantation of anaerobic species in more distal portions.

Finally, two parts should be distinguished within the colon: (a) the ascending colon, which contains mainly gram-positive bacteria, which have the primary role of sugar fermentation, and (b) the descending colon, in which the flora, known as "putrefaction flora," consists mainly of gram-negative bacteria but also some gram-positive bacteria (*Clostridium*, *Bacteroides*).

Table 13 Human Gastrointestinal Flora

	Stomach	Jejunum	Ileum	Colon
Total microbial concentration	$0–10^{3a}$	$0–10^5$	$10^3–10^7$	$10^{11}–10^{12}$
Strict aerobic or facultative anaerobic bacteria				
Enterobacteria	$0–10^2$	$0–10^3$	$10^2–10^5$	$10^4–10^{10}$
Streptococcus	$0–10^3$	$0–10^4$	$10^2–10^6$	$10^5–10^{10}$
Staphylococcus	$0–10^2$	$0–10^3$	$10^2–10^5$	$10^4–10^7$
Lactobacillus	$0–10^3$	$0–10^4$	$10^2–10^5$	$10^6–10^{10}$
Fongy	$0–10^2$	$0–10^2$	$10^2–10^3$	$10^2–10^6$
Anaerobic bacteria				
Bacteroides	Rare	$0–10^2$	$10^3–10^6$	$10^{10}–10^{12}$
Bifidobacterium	Rare	$0–10^3$	$10^3–10^7$	$10^8–10^{12}$
Peptococcus	Rare	$0–10^3$	$10^3–10^4$	$10^8–10^{12}$
Clostridium	Rare	Rare	$10^3–10^4$	$10^6–10^{11}$
Fusobacterium	Rare	Rare	Rare	$10^9–10^{10}$
Eubacteria	Rare	Rare	$10^3–10^5$	$10^9–10^{12}$
Veillonellae	Rare	$0–10^2$	$10^3–10^4$	$10^3–10^4$

[a] Number per gram of intestinal contents.

This conventional theory, associating a region of the colon with a bacterial function and consequently with particularly dominant species[214] has been challenged by the work of Croucher et al.[206] Their studies of human colon biopsies tend to demonstrate that there is no specific location for the various species within the colon.

D. Age and Diet

This change in the flora is closely linked with the maturation of the digestive system, once more highlighting the importance of the reciprocal host-bacteria relationship. Bacteriological examination of the feces shows that diet has little or no effect on the constitution of the dominant intestinal flora.[215]

E. Role and Effect of the Intestinal Flora

"The gastrointestinal tract is a complex ecosystem with characteristics which depend at each moment on a dynamic equilibrium between the host and the native bacteria."[214] Exogenous bacteria also influence all the bacteria within the intestinal flora. Some may be probiotic and others simply commensal, whereas others may be pathogens. The overall effect of the microbial flora on the host is generally evaluated by comparing an axenic animal with a holoxenic animal in which the flora has developed normally. This tool has been found to be most useful in demonstrating the effects of a given species or small group of species on the host.

1. Effect on Physiology of the Intestinal Wall and the Immune Defense System

The intestinal flora modifies the morphology of the mucosa and the rate of turnover and differentiation of epithelial cells. It also follows enterocyte maturation and development

of the velocities in the neonate.[214,216,217] In the axenic animal, Simon and Gorbach[215] observed an increase in the activity of enterocytic enzymes, in particular alkaline phosphatase, disaccharidase, and β-glucosidase. All these studies have demonstrated the importance of the role of the flora, since it determines the uptake of nutrients and permits the formation of the ecological site for other bacteria.

An important role played by the flora is its action in cell maturation observed in the normal development of Peyer's patches. These observations, first made as a result of histological investigation of the intestinal wall, have subsequently been confirmed by numerous studies demonstrating that resistance to various pathogens is conditioned by the presence of a flora. The intestinal bacteria ensure the maintenance of the immune status by providing repeated antigen stimulation throughout the human life span.

2. Bacteria as Nutrient Sources

The bacterial mass of the intestine is itself an important source of nutrients: thiamine, riboflavin, folic acid, vitamin B_{12}, pantothenic acid, short-chain fatty acids, amino acids, and proteins, which are partially absorbed and used by the host.[43,218,219]

3. Metabolic Effects

The bacterial flora produces a very large and varied quantity of enzymes, which are used by the flora itself but also by the host. All the aspects of the intestinal metabolism of the host are influenced by the enzymatic activity of the bacteria it shelters and more particularly the anaerobic bacteria. We will list here some examples of the effects of bacterial metabolism on the host.

Enzymatic Action. These bacteria are able to compensate for enzymatic. deficiencies of the host if they are introduced in a sufficiently large number into the digestive tract. This is the case of lactobacilli ingested with yogurt, which can produce the lactase activity missing in lactose-intolerant subjects.[220]

Detoxification. Another important action of the intestinal bacteria is their involvement in the enterohepatic cycle and the detoxification of numerous substances and drugs.[221] Thus, cholesterol is converted to form coprostanol and the bile salts to form bile acids and then lipocholic acid and other derivatives conjugated with amino acids such as glycine and taurine, facilitating their detoxification and elimination.[218] Rowland and Grasso[222] have investigated the degradation of the *N*-nitrosamines by the intestinal flora.

Production of Harmful Substances. In contrast, some microorganisms produce substances toxic to the host, notably histamine, tyramine, agmatine, cadaverine, ammonium, phenols, *N*-nitrosamines, and bacterial toxins.

4. Tumoral Action

Cancer of the colon is the second greatest cause of death in Great Britain and in the United States. It would appear that 90% of human cancers are due to the environment and could therefore be avoided. Major differences have been observed between cancer risks, but it would seem that neither the place where the population live nor their race is responsible, rather that the etiology of the disease is related to diet.[223] Considerable research has been carried out in an attempt to identify carcinogens.

A diet containing low fiber and high quantities of animal fats appears to promote the onset of cancer of the colon, but no directly active carcinogen has been isolated. Aries

et al.[224] therefore believe that carcinogens may be produced in situ, probably as a result of the enzymatic activity of the bacteria of the digestive flora on a harmless procarcinogen substrate derived from the diet. It is reasonable to think that the intestinal flora could produce or potentiate carcinogens or procarcinogens.

5. Effect of the Anti-infectious Barrier Toward Pathogens

The microbial population of the gastrointestinal tract forms a barrier against proliferation of exogenous pathogens.[224] One explanation may be that the colonization of the endogenous flora maintains the pathogens at a subclinical level by preventing the colonization of the undesirable flora by competition for the substrate or epithelial receptors.[225] This recently discovered role challenges the theory of the anti-infectious barrier effect suggested by Ducluzeau et al.[220] which holds that the barrier effect can be observed only in bacteria belonging to the dominant flora, and that pathogenicity can be effective only above a certain colonization threshold of the invasive bacteria. In fact, the subdominant flora uses the endogenous substrates for its own metabolism, but also for the dominant population, preventing the proliferation of pathogenic bacteria and the adhesion of other organisms. The intestinal microorganisms inhibit the growth of the invasive pathogens by producing organic acids, particularly volatile fatty acids, deconjugating bile acids, which inhibit pathogenic bacteria in their conjugated forms, and producing bacteriocin and volatile acids, which stimulate peristaltism.[227,228]

XIV. BIFIDUM-INTESTINAL RELATIONSHIPS: PROBIOTIC ROLE OF *BIFIDOBACTERIUM*

Tissier[228] observed a close relationship between the immunity of the breast-fed child and his or her specific flora. Bifidobacteria had long been recognized as bacteria with probiotic, nutritive, and therapeutic properties. These properties have subsequently been clearly defined: the ingestion of fermented milk results in stimulation of the immune system.[229,230] The quantities of β-glucuronidase, azoreductase, and nitroreductase formed by the flora are reduced by the ingestion of *Lactobacillus acidophilus*,[231] which has also been shown to be active in the degradation of nitrosamines.[220] A large portion of the world's adult population shows a deficit in galactosidase. Numerous studies have shown that deficient subjects do not show any intolerance toward yogurt.[230] Two explanations can be suggested for this: the activity of bacterial β-galactosidase may persist in the intestine, where a fraction of the lactose present may be metabolized or the ingestion of yogurt may stimulate any intestinal lactase that is still active.

In the case of the bifidobacteria, a probiotic effect can only occur if the bifidobacteria survive their transit through the stomach. Some strains of *Bifidobacterium* are able to resist gastric acidity, and this resistance is increased by the food bolus.[232] The bacterial production of organic acids, particularly lactic and acetic acids, of bacteriocins, and even antibiotics as well as the secretion of enzymes, vitamins, and other growth factors are, together with the stimulation of the immune system and accumulation of specific metabolites, the determining factors in a probiotic action. We can now attribute the functions described below to the bifidobacteria.

A. Adhesion to the Intestinal Epithelium

The adhesion of the bifidobacteria to the epithelial cells permits the formation of ecological niches within which the growth of bacteria is maintained regardless of changes in the habitat, thus enabling them to produce a genuine effect. The bacterial biofilm, bound to the epithelial walls, maintains the mechanism of in situ production of various bacterial metabolites, which themselves have an effect on other bacterial genera and even on the host. In addition, it has a defense function against pathogenic bacteria.

B. Action on the Morphology and Physiology of the Digestive Tract Wall

Bifidobacteria influence the maturation and turnover cycle of the enterocyte, together with the development of the intestinal velocities.[214,215] They are also involved in the degradation and replacement of intestinal mucins.[233–237,145,146] They may also have an action on the immune system appended to the digestive tract.[231]

C. Nutritional Effects

The production of vitamins (B_1, B_6, B_9, B_{12}, and PP), amino acids (alanine, valine, aspartic acid, and threonine), and the fact that they produce only L + lactic acid, which is completely metabolized by humans enhances the nutritional characteristics of the bifidobacteria.[43,218,219]

D. Metabolic Effects

1. Suppression of Lactose Intolerance

Bifidobacteria, unlike *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*, are resistant to biosalts[239] and as a result can have an in situ effect on the metabolism of lactose.

2. Hypocholesterolemic Effect

Several studies have tended to demonstrate a relationship between the presence of a lactic microflora and a reduction in plasma cholesterol. The administration to hyporcholesterolemic human subjects of fermented milks containing very large quantities of *Bifidobacterium* (10^9 bacteria per gram) results in a fall in the total cholesterol from 3 to 1.5 g/mL.[240,241]

The consumption of fermented dairy products could lead to a reduction in serum levels of cholesterol. Bacteria-producing lactic acid also produces hydroxy-methylglutaryl–CoA reductase, which is involved in the synthesis of cholesterol,[242] Rao et al.[243] have shown that the metabolites produced from orotic acid during fermentation of fermented products could be responsible for this hyporcholesterolemic effect. Jaspers et al.[244] have shown that both orotic and hydroxymethylglutaric acids reduce serum cholesterol, whereas uric acid inhibits the synthesis of cholesterol. In vitro, bifidobacteria apparently affect the activity of HMG–CoA reductase.[240] It is difficult to determine the role that should be attributed to the bifidobacteria, and studies are in progress intended to demonstrate the involvement of bifidobacteria in the reduction of cholesterol levels.

3. Deconjugation of Bile Acids, Reduction of Nitrosamines, and Inhibition of the Reduction of Nitrates

Bile acids are secreted into the duodenum in their form of conjugates with glycine or taurine. Most strains of the genus *Bifidobacterium* are able to hydrolyze sodium taurocholate and glycocholate in the colon.[245] The hydrolases involved are constitutive and extracellular.[15]

4. Other Metabolic Effects

The ingestion of milk fermented with *Lactobacillus acidophilus* and *B. bifidum* for a period of 3 weeks has no effect on the production of hydrogen or methane or on fetal β-galactosidase activity, but does increase the activity of fecal β-glucosidase, and these four parameters are good indicators of the fermentation capacity of the colonic flora.[246]

E. Effect of the Anti-Infectious Barrier to Pathogenic Bacteria

It has long been possible to demonstrate close relationships between the probiotic and therapeutic effects of bifidobacteria, but few studies have been carried out in human subjects:

B. longum has a barrier effect against *Escherichia coli* in the axenic rat.[227]

Axenic mice monoassociated with *B. longum* live longer than truly axenic mice after the intravenous or intragastric administration of high doses of viable *E. coli*.[247]

The intestinal flora of leukemia patients is modified by chemotherapy, and bacteria that are usually rare in healthy subjects multiply considerably. This disequilibrium of the intestinal microflora is countered by oral administration of *B. longum*.[248]

In infants suffering from rotavirus-induced diarrhea, the concomitant administration of *B. longum*–fermented milk with the antibiotic treatment results in a reduction in the number of stools and the number of *Bacteroides* and a more rapid regain of weight compared with treatment with antibiotic alone.[249]

Two hypotheses have been advanced to explain this probiotic effect:

1. Prevention of the colonization of the intestine by pathogens by competing for nutrients and for binding sites on the epithelial surfaces.[218,220,227,250]
2. The production of lactic and acetic organic acids during the fermentation of carbohydrates by bifidobacteria, which results in a reduction in the pH of the intestine and consequently inhibits the growth of the undesirable bacteria.[218]

The implantation of bifidobacteria is promoted in infants by breast-feeding. The permanent acidity of the intestinal contents that results from the development of bifidobacteria has a bacteriostatic effect against *E. coli* and gram-negative bacteria.[141]

Acidification has a bactericidal potential, especially against gram-negative bacteria. Acetic acid has a stronger antagonistic effect against gram-negative bacteria than lactic acid, and it is produced in greater quantities by *Bifidobacterium*.[218] This difference appears to be due more to the quantity of undissociated acid than to the type of acid. The pK_a of acetic acid is 4.76, whereas that of lactic acid is 3.86. Acetic acid 8.4% and lactic acid 1.1% are present in an undissociated form at an intestinal pH of 5.8. Acetic and lactic acids are produced in the bifidobacteria in a ratio of 3/2, which results in about 11 times more undissociated acetic acid than undissociated lactic acid. The acidity

stimulates the peristaltic movements of the intestine, which facilitates elimination of any pathogens present.[226,251]

Knocke et al.[252] have investigated the in vitro interactions between *B. adolescentis* and *Bacteroides ovatus*. In a continuously renewed complex medium they observed that the inhibition of *Bacteroides* by the bifidobacteria appears to be due to the production of certain metabolites by the latter.

Yamazaki et al.[6] have investigated the translocation capacity of *E. coli* in the axenic mouse. Intragastric inoculation of *E. coli* at sublethal doses results, weeks later, in the appearance of this strain in the liver, spleen, kidneys, and lungs of monoassociated animals. In contrast, previous implantation of *B. longum* in the axenic mouse allows the animal to survive and results in the disappearance of *E. coli* from all the organisms invaded within one week, although they remain present at high levels within the colon.

The most probable explanation for this phenomenon would be the reinforcement of the immune barriers. *B. longum* may be capable of affecting both humoral immunity and cellular immunity.[227] The production of bacteriocins by bifidobacteria was studied by Meghrous et al.[253] in 13 strains. The antimicrobial substance detected was of a protein type, heat-stable and active at pH values ranging from 2 to 10 versus other gram-positive species including some strains of *Clostridia*.

F. Therapeutic Effects

1. Antitumor Effect

The research so far carried out has been essentially concerned with the direct or indirect antitumor action of streptococci, lactobacilli, and bifidobacteria.[254,255] *B. longum* has a direct inhibitory effect on liver tumors in the mouse.[256] In the mouse, *B. infantis* has an undeniable antitumor effect.[257] The number of tumors developed by mice with an intestinal flora including *E. coli*, *Enterococcus faecalis*, and *Clostridium paraputrificum* is considerably reduced if *B. longum* is present.[256] The antitumor action may be obtained as a result of (a) direct suppression of the procarcinogens,[258] (b) the reduction of indirect suppression of procarcinogens or bacterial enzymes which result in their formation, (c) activation of the host's immune system,[251] and (d) reduction of the intestinal pH.[260]

2. Prevention and Treatment of Other Diseases

In l966 Bamberg[261] successfully treated digestive disorders induced by antibiotic treatment using a lyophilized culture of *B. longum*. Similarly, Haller and Kraüberg[262] and Neumeister and Schmidt[263] obtained similar results in radiation-treated subjects. Bifidobacteria were successfully administered to premature infants with an intestinal flora that had been disturbed by the taking of antibiotics.[264] Seki et al.[265] developed a treatment for constipation in the elderly based on *Bifidobacterium*.

The ingestion of milk fermented with *B. longum* is successful in regularizing digestive transit in pregnant women (reduced abdominal ballooning, diarrhea-type phenomena, or constipation).[7] The intestinal flora and health status of children suffering from diarrhea were restored more rapidly after the ingestion of milk fermented with *B. breve*.[266] The ingestion of milk fermented with *B. longum* can play not only a preventive role in healthy subject, by maintaining high levels of bifidobacteria in the flora, thus preventing diarrhea and constipation, but also a therapeutic role in patients suffering from diarrhea.[267] The intestinal disorders in 34 Soviet cosmonauts were successfully treated by the ingestion of bifidobacteria.[268]

This nonexhaustive review of the beneficial effects of bifidobacteria demonstrates that in all cases it was bifidobacteria of human origin and more particularly *B. longum* that had been used.

XV. CONCLUSION

The current use in fermented milks of probiotic bacteria and of bifidobacteria in particular presumes that the user will take all necessary precautions with regard to the following aspects.

A. Choice of Species

This is the most important factor if it is intended that the product should have genuine probiotic qualities. Since *B. dentium* is recognized as being pathogenic, eight species could theoretically be used.

B. Strain Identification

B. bifidum, *B. breve*, *B. infantis*, and *B. longum* constitute no danger, but other bifidobacteria in this group could be confused with *B. dentium* if genetic methods are not used to identify the strain (Table 11).

C. In Vitro and In Vivo Verification of the Probiotic Potential of the Strain

This potential can only be demonstrated through clinical and nutritional studies, and no strain has all the qualities demonstrated for the genus *Bifidobacterium* as a whole. In this field, the choice must still be based on the probiotic activities desired.

D. Technological Potential

The choice in this field must take into account several factors such as the acetate/lactate ratio, the relationships of the bifidobacteria with other species in the product, the tolerance of the strain of acidity and above all of oxygen. Furthermore, the distribution conditions for these products must be such as to ensure the survival of the bifidobacteria under good physiological conditions.

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3

An Update on Probiotic Bifidobacteria

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I. INTRODUCTION

Research and commercial interest in the genus *Bifidobacterium* has flourished in recent years, driven almost entirely by their potential health benefits in probiotic functional foods. In 1990 there were approximately 30 publications in the international literature focusing on bifidobacteria. In 2002 there were more than 10 times that number. The previous chapter by Jean Ballongue provides an historical perspective on the discovery of bifidobacteria, their basic physiology, and an overview of the beginnings of a new wave of interest in these organisms as probiotics. Since Ballongue's chapter was published in 1998, there have been numerous changes within *Bifidobacterium* taxonomy and the development of new applications and food technologies. A fundamental switch from culture-based, phenotypic examination of microbial diversity to the application of culture-independent molecular techniques has provided new insights into the ecology of bifidobacteria within the intestinal tract. Exciting advances have been made in demonstrating the efficacy of probiotic bifidobacteria in human health, including roles in the treatment of allergy and the maintenance of remission in inflammatory bowel disease. New therapeutic applications for bifidobacteria have emerged, such as their use to deliver targeted gene therapy to hypoxic tumors. This chapter provides an update on our current knowledge of bifidobacteria as probiotics. It focuses on recent advances in food technology, our understanding of the ecology of bifidobacteria in the human intestinal tract, and emerging evidence for clinical benefits and mechanisms of probiotic action.

II. WHY BIFIDOBACTERIA?

Lactic acid bacteria have a long history of use in fermented foods. However, the question is often raised as to why bifidobacteria have joined lactobacilli as a target for probiotic

research and commercial application. From an historical perspective, there were some sound scientific and technological reasons for the initial development of interest in this genus. First among these was that they were known to constitute a major population group within the human colonic microbiota.[1] Interest in reinforcing the numbers of these bacteria in the intestinal tract stemmed from studies showing that bifidobacteria constituted the numerically dominant microbial population in the intestinal tract of infants who were fed breast milk, whereas infants who were fed using cow's milk–based formulas developed a mixed microbiota, including higher levels of potentially deleterious organisms.[2,3] Additionally, the levels of bifidobacteria were shown to decline in the elderly, and were replaced by putrefactive bacteria, especially clostridia and enterobacteria (see Chapter 2). Like lactobacilli, bifidobacteria were considered desirable, health-promoting bacteria, with a saccharolytic and acidogenic physiology, and without involvement in putrefying or toxigenic reactions or pathogenicity. Hence, the consumption of live bifidobacteria to maintain or restore a population of "healthy" intestinal bacteria was popularized in a similar way to lactobacilli before them.

Technological considerations also facilitated the adoption of bifidobacteria as probiotics. Bifidobacteria produce lactate and acetate during sugar fermentations, without gas formation, and, for most strains, without compromising the organoleptic qualities of fermented dairy foods. Many species grow well using lactose as a carbon source, and so they can be used in fermented dairy products, the traditional food vehicle used for probiotic lactobacilli. With the theory that two is better than one, probiotic bifidobacteria now appear alongside lactobacilli in a large proportion of probiotic functional foods. The science to evaluate the theoretical benefits of the consumption of bifidobacteria has largely lagged behind their commercial application.

III. TAXONOMY

As for most bacterial genera, the application of molecular techniques to determine relatedness of bacteria at the genome level has led to some recent reclassifications of species within the genus *Bifidobacterium* (Table 1). Notably, *Bif. infantis* and *Bif. suis* have been amalgamated into *Bif. longum*;[4] *Bif. lactis* has been absorbed into *Bif. animalis*,[5] while *Bif. denticolans* and *Bif. inopinatum* have been reclassified into new genera.[6] The names *Bif. infantis*, *Bif. suis*, and *Bif. longum* are recognized as heterotypic synonyms.

Table 1 Changes in the Taxonomy of Bifidobacteria

Old designation	New designation	Recent additions to the genus
Bif. denticolans	*Parascardovia denticolens*	*Bif. coryneforme*
Bif. globosum	*Bif. pseudolongum* subsp. *globosum*	*Bif. gallicum*
Bif. infantis	*Bif. longum*	*Bif. gallinarum*
Bif. inopinatum	*Scardovia inoponata*	*Bif. merycicum*
Bif. lactis	*Bif. animalis*	*Bif. ruminantium*
Bif. suis	*Bif. longum*	*Bif. saeculare*
		Bif. scardovii
		Bif. thermacidophilum

A number of new species of bifidobacteria have also been isolated and characterized (Table 1). Among these, *Bif. thermacidophilum*[7] stands out from other *Bifidobacterium* species. As its name suggests, it can grow at elevated temperatures (49.5°C) and at relatively low pH (4.0).

Taxonomy is important not only from scientific, evolutionary, and ecological points of view, but also from regulatory, safety, and marketing standpoints. Taxonomic classification should be included as an early step in the development of any new probiotic strain. Demonstrating a safe history of use in foods facilitates passage of probiotic ingredients through food regulatory frameworks, and identifying a new probiotic strain at the species level is important in assessing its relative risk. In the case of bifidobacteria, species other than *Bif. dentium*, which is associated with dental caries, are generally regarded as safe.[8]

In the past there has been substantial discrepancy between the *Bifidobacterium* species designated on a product label and the species found in the product itself.[9,10] Manufacturers of probiotic dairy products appear to have improved their attention to correct taxonomy in recent years,[11] if only by limiting designations to the genus and strain level. There is still considerable room for improvement,[12] and the problem appears to still be particularly widespread in freeze-fried probiotic preparations[11] and animal probiotic products.[13,14] The constant flux in species designations can cause headaches for commercial suppliers, but since probiotic attributes vary within species and must be defined at the strain level, a strain designation is the primary identification promoted for many probiotics.

The reclassification of *Bif. lactis* to *Bif. animalis* is probably the most contentious of the new changes in taxonomy. Strains formerly classified as *Bif. lactis* are the most commonly used bifidobacteria in fermented dairy probiotic products,[11] and the name *Bif. lactis* is rather more palatable to marketers of probiotics for human consumption than *Bif. animalis.* Strains within the *Bif. lactis/animalis* group are considerably more acid-, oxygen-, and thermotolerant than most human intestinal *Bifidobacterium* species (Figs. 1, 2) and technologically suited to fermented dairy products. On the basis of distinct clusters from PCR methods targeting the 16S–23S internally transcribed spacer region of *Bif. animalis* and *Bif. lactis*, the separation of *Bif. lactis* from *Bif. animalis* at the subspecies level has been proposed.[15]

The application of molecular techniques to characterize currently uncultivable bacteria will undoubtedly lead to the discovery of many new species and genera of bacteria in a range of habitats. In the complex milieu of the intestinal tract of humans and animals, there are almost certainly new species awaiting discovery, and the taxonomic map of the bifidobacteria can be expected to undergo further changes in the coming years.

IV. SAFETY

Except for *Bif. dentium* (dental caries), bifidobacteria are regarded as safe.[8] A number of bifidobacteria now have a long history of safe use as dietary adjuncts, *Bif. adolescentis*, *Bif. animalis/Bif. lactis*, *Bif. bifidum*, *Bif. breve*, and *Bif. longum/infantis* have GRAS (generally regarded as safe) status.[16] After more than 20 years of use in probiotic foods and investigation in a large number of animal studies and in human feeding trials, deleterious effects have yet to be reported for bifidobacteria. Human feeding studies have been performed without reports of adverse health effects in age groups ranging from infants[17,18] to the elderly[19–22] and in healthy individuals and those with intestinal disease.[23–26]

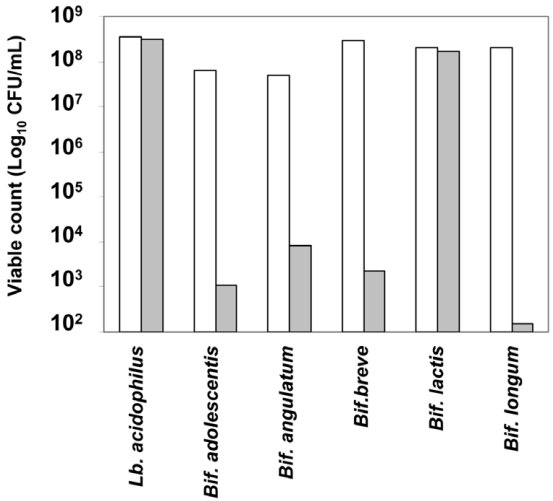

Figure 1 *Bifidobacterium lactis* is considerably more tolerant to acidic conditions than species indigenous to the human gastrointestinal tract. This figure represents viable counts of bacteria before (□) and (■) after incubation in HCl/KCl at pH 2.0 for 105 min at 37°C. (Modified from Ref. 39.)

Reports of human bacteremias involving bifidobacteria are rare. In the rare cases of bacteremia where bifidobacteria have been found, the subject has been predisposed to infection by surgical trauma or underlying health disorders, and the bifidobacteria have formed part of a mixed culture bacteremia involving other usually benign commensal bacteria.[27] The rarity of reported infections and inadequate taxonomic characterization

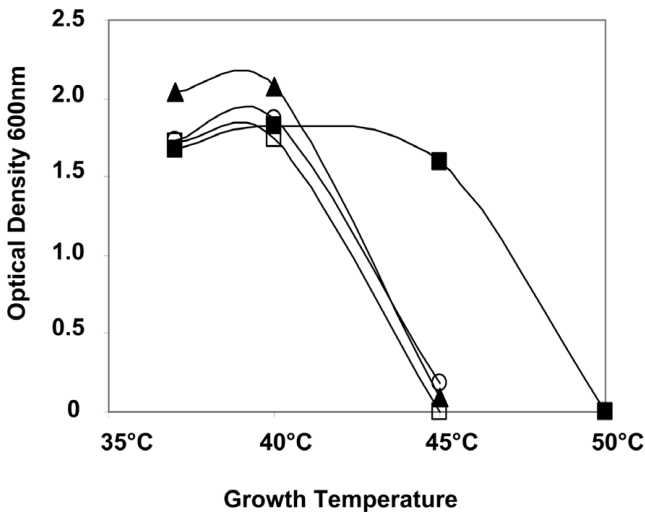

Figure 2 *Bifidobacterium lactis* is able to grow at higher temperatures than *Bifidobacterium* species indigenous to the human gastrointestinal tract. ■ *Bif. lactis*; ○ *Bif. adolescentis*; □ *Bif. longum*; ▲ *Bif. breve*. (Modified from Ref. 39.)

of offending isolates has precluded any associations being made between particular species and human bacteremias. The low incidence and predisposing circumstances of bacteremias involving bifidobacteria, when combined with the long and safe history of use of some strains in foods, supports the conclusion that bifidobacteria are generally safe for probiotic use. However, this does not mitigate the need for adequate safety testing of new probiotic *Bifidobacterium* strains before they are used commercially. Surveying of the probiotic bifidobacteria used in yogurts and lyophilized products has demonstrated regular discrepancies between species labeled and those in the product, with yogurt being dominated by *Bif. lactis/animalis*.[9,11] Therefore, the number of species with a safe history of use in foods may be lower than the history of product labeling would suggest. The application of culture-independent methods of investigating microbial diversity in nichés within the host will lead to the identification of new species that require characterization from a safety viewpoint. One example is a potentially novel *Bifidobacterium* species identified in deep caries that may play a role in disease.[28] A number of articles discuss safety tests that should be applied to new probiotic isolates.[8,27,29] (See Chapter 19 for further details on the safety assessment of probiotics.)

V. TECHNOLOGY

Probiotics are believed to elicit their beneficial health effects when viable, although this dogma is being increasingly challenged.[30,31] Whether viability is an essential requirement for all health effects elicited by probiotics is an intriguing question that demands further investigation. Currently, however, manufacturers aim to maintain a high level of probiotic culture viability throughout the shelf life of their products. Not only viability, but also functionality must remain stable during manufacture, formulation, storage, and intestinal delivery. Since bifidobacteria are strict anaerobes and generally less acid tolerant than lactobacilli, their use in functional foods provides greater technological challenges.

Fermented dairy products are still the major food vehicles in which probiotic bifidobacteria are delivered. A number of parameters, including interactions with other starter and probiotic strains, salts, sugars, and flavoring and coloring compounds, can influence the survival of bifidobacteria in these products.[32–36] However, oxygen and pH exert perhaps the greatest influence on *Bifidobacterium* survival during storage. Oxygen sensitivity can be addressed by strain selection and with appropriate packaging techniques and materials.[37] The relative oxygen sensitivity of a range of *Bifidobacterium* strains has been reported by Beerens et al.,[38] and in general species of animal origin are more oxygen tolerant than those isolated from humans.

The physiological mechanisms behind the acid tolerance of *Bif. animalis/lactis* compared to human intestinal isolates remains to be investigated and may provide important clues to improving the survival of pH-sensitive strains. *Bif. lactis* can survive well in fermented dairy products throughout their shelf life if the pH is prevented from dropping below 4.1.[39] This is sometimes achieved by limiting fermentation by traditional yogurt starter cultures or by omitting starter species, although the inclusion of *Lactobacillus delbrueckii* subsp. *bulgaricus* is mandatory in yogurts in many countries.

Most human intestinal *Bifidobacterium* isolates are more challenging to grow and maintain higher viability during product storage than *Bif. animalis*, and technological approaches such as immobilization, microencapsulation, and induction of stress proteins have been employed to improve stability. The outcomes of some of these strategies are discussed later in this section.

A. Food Applications Other Than Fermented Dairy Products

Bifidobacteria have now been successfully applied to a range of food matrices beyond dairy yogurt and fermented milk. To simulate the bifidogenic effect of human breast milk, bifidobacteria and bifidogenic oligosaccharides are now included in some powdered infant formulas.[40,41] They have been used in fermentations of goat's,[42] sheep's, and camel's milk[43] and in fermented soybean[44–46] and oat-based[47,48] products. Bifidobacteria have been reported to grow and survive in a variety of cheeses[49–52] and remain viable in ice cream[53] and in frozen yogurt.[54] Attempts to maintain high viability of bifidobacteria in coleslaw[55] and a table spread[56] have proved less successful.

One interesting application of these bacteria has been as a biocontrol agent in fermented meat. A *Bif. lactis* strain incorporated into a Hungarian salami was reported to inhibit the growth of *Listeria monocytogenes* and *Escherichia coli* strain 0111.[57]

B. Microencapsulation

A number of microencapsulation technologies to protect bifidobacteria against acidic conditions have been developed. Most have been only partially successful. The technical hurdles in the application of microencapsulation for probiotic bifidobacteria are not insignificant. The demands include protection against oxygen and other environmental stresses during product formulation and storage; protection against low pH, proteases, and bile during gastric intestinal transit; efficient release of the bacteria within the gastrointestinal tract; and all with an encapsulating material that is inexpensive, stable, food-grade, and with suitable sensory properties. Alginate,[58–65] carrageenan,[66,67] modified starch,[68] gellan-xanthan,[69] and cellulose acetate phthalate[70] have all been trailed. In laboratory models simulating the acidic conditions in the stomach, encapsulation with alginate, carrageenan, and modified starch have generally failed to substantially improve the survival of acid-sensitive bifidobacteria. More encouraging results have been reported for improved survival in food products, but it is questionable if the level of improved viability could justify the increased production costs in commercial food applications. Another impediment to the application of some microencapsulation techniques in certain food matrices is the imposition of deleterious effects on sensory properties. Those reported have included imparting a grainy texture[67] and stimulating production of off-flavors, presumed to be due to mass transfer–induced nutrient limitation effects on metabolism.[65]

However, the story of microencapsulation of bifidobacteria is not all doom and gloom. Microparticles manufactured using alginate and poly-l-lysine and particles produced using cellulose acetate phthalate in spray drying have been reported to significantly improve survival of bifidobacteria in acidic environments.[61,70] Microencapsulation has also been shown to provide a beneficial impact on sensory parameters in yogurt by preventing excessive acidification, while at the same time improving viability.[66] Microencapsulation may yet prove to be a valuable approach to maintain the viability of environmentally sensitive bifidobacteria in long shelf-life food products.

C. Induction of Stress-Response Proteins

Perhaps the most judicious approach to improving the tolerance of bacteria to environmental stresses is by preconditioning cultures through induction of shock proteins. The presence of the heat shock protein gene dnaK has been demonstrated in *Bif. longum*,

Bif. adolescentis, and *Bif. breve*.[71] Such stress-response proteins in bifidobacteria might be exploited to promote survival of strains during intestinal transit. Preconditioning with bile salts was shown to provide improved protection against lethal concentrations of bile for *Bif. longum* and *Bif. adolescentis.* As in other bacteria, cross-protection was afforded by the stress-response proteins since salt pre-treatment of a *Bif. longum* strain resulted in increased tolerance to both freeze-thawing and lethal heat stress. Inducing stress proteins during fermentation and maintaining their presence downstream may contribute to improved survival of environmentally sensitive bifidobacteria during manufacture, storage, and gastrointestinal transit.

D. Fermentation and Drying

Bifidobacteria generally grow well in industrial media that support the growth of lactobacilli, including whey-based media supplemented with yeast or beef extract.[72] Prehydrolysis of media with proteases often improves fermentation yields,[73] and consideration of the carbon substrate is important since not all strains can utilize lactose. Companion cultures, including lactobacilli and propionibacteria, have also been shown to increase *Bifidobacterium* yields in fermentations in a strain-dependent manner.[74,75]

Occasionally intestinal isolates prove quite fastidious with respect to their growth environment. One example is the apparent requirement of some strains for high cell density for growth. A too dilute inoculum or even gentle stirring of the cultures, even under anaerobic conditions, inhibits the growth of such strains.

Freeze-drying remains the most commonly employed method of drying bifidobacteria for commercial use. However, spray-drying has been used successfully for some strains when carriers such as gelatine gum arabic, or starch were added and relatively low air outlet temperatures (50°C) were used.[61,68,76] The suitability of spray drying for bifidobacteria is highly dependent on the robustness of individual strains.

E. Bioconversions

The metabolic activities of bifidobacteria have been exploited to produce bioactive compounds during fermentations that potentially increase the health functionality of the food products. Examples include the production of folic acid[77] and oligosaccharides in fermented milk and yogurt,[78] transformation of isoflavone phytoestrogens in soy milk,[45] synthesis of conjugated linoleic acid from free linoleic acid,[79] and production of bioactive peptides with potential antihypertensive effects.[51]

VI. ECOLOGY OF BIFIDOBACTERIA WITHIN THE HUMAN GASTROINTESTINAL TRACT

The application of bifidobacteria as probiotics has provided fresh impetus for research aiming to provide an understanding of the ecology of these bacteria within the human intestinal tract. Knowledge of the interactions between the microbiota and the health and disease of the host is an essential prerequisite for a mechanistic understanding of probiotic efficacy.

Recent years have seen a surge in the development of the molecular tools needed to investigate bacterial population dynamics in complex ecosystems. Until the mid-1990s most investigations of intestinal microecology had relied on culture techniques to isolate the bacteria. The complexity of the intestinal microbiota was still recognized, however,

and it was estimated to contain more than 400 species dominated by perhaps 30–40.[80] The emergence of culture-independent, genetic methods to examine bacterial diversity has revealed that a significant fraction of the microbiota [up to 60%][80] remains uncultivable and that the intestinal ecosystem is far richer than was previously believed.[81] New taxa continue to emerge, and the number of bacterial species identified within the intestinal microbiota may eventually exceed 1000.[81]

The 16S ribosomal RNA (rRNA) gene has become an important tool in classification, enabling investigation of microbial diversity at both the genus and species level.[81] The discriminatory power of genetic fingerprinting techniques has allowed even deeper examination of bacterial diversity at the strain level.[82–85] Genetic techniques to study intestinal microbial ecology have been eloquently reviewed by Vaughan et al.,[81] Tannock,[84] and McCartney.[86]

Understanding the role of bifidobacteria within the intestinal microbiota and their interactions with diet and with the host is an enormous task, made more onerous by the difficulties in sampling within the intestinal tract. We are only at the very beginning of developing this knowledge, but can expect rapid advances in coming years with the application of high-throughput genomic technologies to this task.

A. Where Are Bifidobacteria Within the Intestinal Tract?

Indigenous bacteria are not distributed evenly throughout the gastrointestinal tract but are found at population levels and in species distributions that are dictated by the environmental conditions that are characteristic of specific regions of the gut.[87] The stomach and proximal small intestine contain relatively low numbers of bacteria (10^3–10^5 bacteria/mL) because of the low pH and rapid flow in this region. Bacterial numbers and diversity increase through the intestine, reaching 10^8 bacteria/mL in the ileum and 10^{10}–10^{11} bacteria/g in the colon.[87]

In the feces of healthy adults, bifidobacteria are generally found in the order of 10^8–10^{10} bacteria/g.[81,85,88,89] Due to the difficulties involved in obtaining samples, there have been few reports of *Bifidobacterium* ecology at different sites in the human intestinal tract. Marteau et al.[90] investigated the differences in total *Bifidobacterium* numbers (and other bacteria) in samples collected from the cecum of healthy volunteers via an intestinal tube and compared the cecal population to that found in feces. Using culture methods, the bifidobacterial population in feces (8×10^8 bacteria/mL) was found to be 100-fold larger than in the cecum (5×10^6 bacteria/mL). However, in terms of the total bacterial population, investigated using 16S RNA probes, the relative proportion of bifidobacteria declined from 5.8% of total RNA in the cecum to 3.2% in feces (a figure in agreement with other estimates of the fecal bifidobacterial population using culture-independent methods[91–94]).

In addition to the different anatomical sections of the intestinal tract, distinct microhabitats also exist within each gut compartment. These include the intestinal lumen, the unstirred mucus layer of the epithelium, the deep mucus layer within the intestinal crypts, and the surface of mucosal epithelial cells.[87] Many bifidobacteria adhere to human intestinal mucus in vitro,[95–98] and introduced probiotic strains have been found both associated with the mucosa throughout the colon and in the lumen.[25] Within the lumen, particulate matter, including dietary fibers and other undigested food particles, also supply sites for bacterial colonization.[99] Many bifidobacteria adhere well to resistant starch,[100] and it is likely that they colonize particulate substrates in the colon. The site of intestinal

colonization may be important in the mechanism of health benefits imparted by probiotic bifidobacteria, such as colonization resistance and immunomodulation, but further work on the molecular bases for health mechanisms is required before such assessments can be made.

B. Which Species Are Present and How Does the Population Change with Age?

1. Infants

Bifidobacteria colonize the human intestinal tract during or soon after birth and in breast-fed infants eventually dominate the microbiota, forming up to 90% of the total fecal bacteria.[101] The dominance of bifidobacteria is induced by bifidogenic components in breast milk, mainly oligosaccharides.[41,101] The *Bifidobacterium* species most commonly isolated from breast-fed infants are *Bif. bifidum*, *Bif. breve*, and *Bif. longum/infantis*.[87,102]

2. Adults

The proportion of bifidobacteria in the colonic microbiota drops following weaning and the introduction of solid food. In adults, they have been reported to account for 1–5% of the total bacteria in feces in studies using molecular techniques that account for the uncultivable microbiota.[81,90–94] The numerically dominant species in the intestinal tract also changes with age. In adults, *Bif. adolescentis*, *Bif.catenulatum/pseudocatenulatum*, *Bif. bifidum*, and *Bif. longum* are the most frequently reported species, with considerable variation between individuals.[89,94,102–104]

3. Elderly

Early studies using culture methods reported that *Bifidobacterium* levels decreased as a proportion of the total fecal microbiota in elderly Japanese.[1] This finding has only recently been readdressed using modern bacteriological and molecular techniques. A study from the United Kingdom[105,106] confirmed the earlier Japanese observation[1] of a drop in *Bifidobacterium* numbers in the elderly using viable counting procedures, 16S rRNA analysis, and community cellular fatty acid profiles. In contrast, the reported fecal *Bifidobacterium* numbers were not abnormally low in two independent studies of elderly Italians,[107,108] or in a Dutch study by Harmsen et al.[93] comparing the fecal bifidobacterial levels from different age groups using fluorescent in situ hybridization (FISH). Harmsen et al.[93] found that bifidobacteria declined from 68% of total bacteria in the feces of breast-fed infants to 5.6% of total bacteria in young children (1–10 years) and 2.5% in adults (25–55 years) and actually increased to 8.5% in elderly subjects (75–95 years). The influence of diet, cultural, and genetic variations on intestinal ecology are still poorly understood and may result in regional differences in intestinal microecology.

In addition to a drop in bifidobacterial numbers, Hopkins et al.[106] observed that the diversity of *Bifidobacterium* species in the intestinal tract decreases with age. Although considerable variation exists between individuals, *Bif. adolescentis* and *Bif. longum* appear to be the most common intestinal species in the elderly,[96,108] with *Bif. bifidum* [108] and *Bif. angulatum* also reported.[106] One of potentially many mechanisms for this change in the size and composition of the intestinal bifidobacterial population in the elderly may be altered mucosal adhesiveness. Intestinal mucus isolated from the elderly supports in vitro adhesion of bifidobacteria relatively poorly compared to mucus isolated from infants and

healthy adults.[95] Additionally, bifidobacteria isolated from the elderly, especially strains of *Bif. adolescentis*, bind poorly to intestinal mucus compared to bifidobacteria from adults,[96] perhaps reflecting a change in selective pressures for mucosal colonization.

C. How Stable Is the *Bifidobacterium* Population Over Time?

In infants, the intestinal microbiota is unstable and developing and through childhood to adulthood develops increasing complexity and stability.[81,109] In adults, the bacterial population of the colon at the genus and species level appears to be very stable over time.[80,81,109,110] Satokari et al.[89] used PCR-DGGE (PCR-denaturing gradient gel electrophoresis) to follow the *Bifidobacterium* species diversity in five healthy adults over a 4-week period. No substantial changes in the species composition or relative intensity of bands were observed, indicating that the *Bifidobacterium* population remained stable at the species level. Similar results were reported by Tannock,[80] including a study in which the fecal bifidobacterial population of an individual was monitored for 8 months using species-specific PCR-DGGE.[110]

At the strain level, differences in *Bifidobacterium* population stability were observed between two adults monitored for 12 months.[82] Further analyses of larger sample sizes over long periods at a strain and species level are required to provide a better picture of ecological stability at the strain level.

D. Are My Bifidobacteria Different from Yours?

The application of molecular techniques to profile the complex microbial communities has revealed that each person has a unique intestinal microbiota at the community, genus, and species level.[109,110] PCR-DGGE analysis of the composition of *Bifidobacterium* species in the feces of different individuals has shown that each person has a unique pattern.[80,89] It appears that we all harbor our own particular combination and proportion of *Bifidobacterium* species. When differences between individuals' intestinal bifidobacteria have been investigated at the strain level, each person has generally harbored unique multiple strains.[82,83,85] Despite these differences in taxonomy, it is likely that the functionality and biochemistry of the intestinal bifidobacterial population as a whole differs little between individuals.[110]

This uniqueness of species and strain combinations may have important implications for the introduction of "foreign" probiotic strains into the intestinal tract of adults with a developed and stable intestinal microbiota. It opens debate into the relative merits of the prebiotic approach of stimulating proliferation of the native intestinal bifidobacteria versus probiotic effects within different age groups.

E. Can Diet Influence the *Bifidobacterium* Population Dynamic?

The example of differences between the size and composition of the intestinal *Bifidobacterium* populations in breast milk-fed and formula-fed infants clearly demonstrates that diet can influence bacterial population dynamics during the early maturation of the intestinal microbiota. This effect is elicited largely by oligosaccharides within human breast milk[87,101] and can be simulated through the supplementation of cow's milk–based formulas with nondigestible oligosaccharides (NDOs).[41,111] The bifidogenic effect of dietary NDOs can be replicated in adults, although to a lesser extent. Consumption of a range of oligosaccharides can induce 10- to 100-fold increases in *Bifidobacterium* num-

bers in the intestinal tract of adults.[112] Bifidogenic oligosaccharides form part of a class of dietary supplements called "prebiotics,"[113] which are discussed in more detail later in this chapter.

Although bifidobacterial numbers can be increased using prebiotics, the apparent stability of the intestinal bifidobacterial population dynamic in adults suggests that day-to-day fluctuations in diet have little impact on the species composition. Feeding of 8 g/day of galacto-oligosaccharides to healthy adult volunteers did not result in marked changes in the composition of their intestinal bifidobacterial population.[114–116] However, a prominent bifidogenic effect was also not observed in this feeding trial, probably due to the high initial level of bifidobacteria. It remains to be seen if prolonged consumption of dietary carbohydrates that are selectively fermented by only a narrow range of *Bifidobacterium* species, such as arabinoxylans,[117] can alter the composition of *Bifidobacterium* species within the intestinal tract.

F. Is the Intestinal *Bifidobacterium* Population Dynamic Associated with Health and Disease?

The growing recognition of the importance of the intestinal microbiota to the healthy maturation of the host's immune system, including the development of tolerance to dietary antigens, has stimulated investigations of differences between the intestinal microbiota of atopic and healthy infants.[118–124] Differences have been observed in the respective intestinal bacterial populations in allergic and healthy infants, including within the bifidobacteria. The total numbers of bifidobacteria are lower in feces of allergic infants,[118,119,122] and the *Bifidobacterium* species composition in allergic infants is more adult-like, being dominated by *Bif. adolescentis*.[98,125] The *Bifidobacterium* isolates from allergic infants have also been shown to be less adherent to intestinal mucus than isolates from healthy infants.[98] However, differences in the microbial ecology of healthy and allergic infants have only been investigated for a narrow range of organisms, and cause/effect links between these observed differences remain to be established. Significant differences were not observed in the *Bifidobacterium* populations of milk-hypersensitive and healthy adults.[126]

The size of the intestinal *Bifidobacterium* population has also been shown to be relatively small in subjects afflicted with inflammatory bowel disease (IBD),[127] irritable bowel syndrome (IBS),[128] and in elderly people with *Clostridium difficile*–associated diarrhea.[129] Direct cause/effect links between these diseases and a diminished bifidobacterial population remain to be established, but probiotic intervention may prove beneficial in the control of these conditions.

Although the level of bifidobacteria in the feces of healthy adults is normally in the order of 10^9 bacteria/g,[80,81,85,89] it appears that a proportion of healthy adults have intestinal bifidobacterial populations several orders of magnitude lower.[89,102,130,131] It is yet to be determined how the total number of bifidobacteria within a stable microbiota influences the long-term health of the human host, and if in individuals with naturally low levels of bifidobacteria, other organisms with similar activities occupy the same niche.

G. How Do Antibiotics Affect the *Bifidobacterium* Population in the Gut?

Administration of certain antibiotics is well known to perturb the intestinal microbial population dynamics. The long-term effects on the composition of the bifidobacterial

microbiota following administration of antibiotics to which they are sensitive differs with the antibiotic used. Following 6 days of treatment with oral rifampicin and streptomycin, the *Bifidobacterium* strains collected in the dominant fecal microbiota of five human subjects were, in most cases, different from before treatment.[85] In contrast, 3 months after 8-day treatment with oral amoxicillin-clavulanic acid (Augmentin), the bifidobacterial strain composition remained largely unchanged from before treatment.[85]

The impact of antibiotics on the microbiota of children may be particularly critical if it impacts on immune development. Prolonged antibiotic treatment in early childhood has been significantly associated with a subsequent history of asthma.[132] Tannock[80] reported the results of a trial in which the composition of the intestinal microbiota of 10 children was examined before and after treatment with antibiotics. In 7 out of 10 children, antibiotic treatment resulted in a marked change in the level and species composition of the bifidobacterial population, as determined by genus-specific PCR-DGGE. Probiotic supplementation following antibiotic treatment may have a role in preserving correct immune development in infants in addition to maintaining colonization resistance.

H. What Happens When Probiotic Bifidobacteria Are Introduced into the Intestinal Tract?

The theoretical bases for many of the anticipated probiotic effects of bifidobacteria rely on the bacteria being viable in the intestinal tract. Molecular strain fingerprinting techniques[18,114–116] and the use of antibiotic-resistant mutants[25,133] have enabled tracking of ingested strains within the complex intestinal microbiota. It is clear that selected probiotic bifidobacteria do survive transit through the stomach and small intestine and can be recovered in feces. In most cases the strain persists only transiently in the intestine,[18,25,46,114–116,133] but the duration of persistence in the intestinal tract is dependent on the individual and almost certainly the host–bacterial strain compatibility. Fujiwara et al.[133] recovered a strain of *Bif. longum* in the feces of some individuals up to 30 days after cessation of probiotic ingestion. Through investigations of colonic biopsies obtained during colonoscopies, von Wright et al.[25] determined that a probiotic *Bif. infantis* strain could be isolated from the mucosa throughout the length of the colon.

One contentious question has been the importance of host specificity in probiotic function. *Bif. lactis/animalis* is the predominant *Bifidobacterium* species currently used in probiotic foods, but is not an autochthonous member of the human intestinal microbiota. It is also taxonomically distant from human intestinal species.[134] However, it adheres well to human intestinal mucus in vitro[97] and transiently colonizes the human intestinal tract following consumption.[18,114–116] Human intestinal species were shown to adhere better to human intestinal mucus than to bovine intestinal mucus,[97] but the question of the importance of mucosal adhesion and strain origin in colonization and probiotic activity of bifidobacteria remains unresolved.

The impact of introducing probiotic bifidobacteria on the already resident intestinal *Bifidobacterium* population dynamic and on the composition of the wider bacterial community is of considerable interest. It would be undesirable for an introduced probiotic to perturb the resident microbiota. PCR-DGGE analysis of the intestinal microbiota of children before and after consumption of *Bif. lactis* for 12 weeks revealed no major disturbances of the dominant bacterial groups in the intestine.[18] Using genus-specific PCR-DGGE, Satokari et al.[115] also observed no effect on the qualitative composition of the indigenous *Bifidobacterium* population after consumption of *Bif. lactis.*

Malinen et al.[116] examined the same samples using PCR-ELISA and observed a decrease in *Bif. longum* numbers while *Bif. lactis* was present in the intestinal tract. *Bif. longum* numbers returned to normal following cessation of the probiotic feeding, which could suggest that *Bif. lactis* transiently supplanted a portion of the indigenous *Bif. longum* population. However, it appears that ingestion of *Bif. lactis* does not significantly alter the intestinal bifidobacterial population of healthy adults in the long term.

Just as important as bacterium numbers are the biochemical and functional activities of probiotics within the intestinal tract and, most critically, their interaction with the host. Advances in functional genomics and investigation of in situ mRNA expression may provide insights into the activities of bifidobacteria within the intestinal tract. The genomes of *Bif. breve* and *Bif. longum* are now being sequenced,[135] and the genomics approach is beginning to yield information increasing our understanding of the physiology of these organisms.[136] Host responses to probiotics and their role in disease prevention are also being uncovered. An example is the induction of intestinal mucin gene expression in the cell lines HT-29 and Caco-2 by lactobacilli and the subsequent suppression of pathogen adhesion to the intestinal epithelial cells.[137,138] The unfolding genomics/proteomics revolution is set to dramatically accelerate our understanding of host-microbe interactions and provide new insights into probiotic mechanisms.

VII. PREBIOTICS

The consumption of live bacteria is not the only strategy to increase the size of the *Bifidobacterium* population in the intestinal tract. Interest in bifidogenic factors developed from attempts to replicate the bifidogenic effect of human milk oligosaccharides in infant milk formulas. A number of nondigestible oligosaccharides have now been demonstrated to act as bifidogenic factors.[112] Since they promote the growth of probiotic bacteria, factors that specifically promote the growth or activity of probiotic organisms within the intestinal tract have been termed "prebiotics."[113] There is an obvious synergy between probiotics and prebiotics, and products containing both have been called "synbiotics".[113]

There is now little doubt from the volume of animal and human studies investigating prebiotic effects that some indigestible oligosaccharides can induce a significant proliferation of bifidobacteria in the intestinal tract of individuals with initially low bifidobacterial numbers.[112,139] They are used, often in synbiotic combinations with bifidobacteria, in cow's milk–based infant formulas[111] and may be useful in reinforcing bifidobacterial numbers in the elderly.[140] To date, the most consistent evidence accumulated for prebiotic effects has been for fructo-oligosaccharides, although galacto-, gluco-, xylo-, isomalto-, and soybean oligosaccharides have also been shown to increase colonic bifidobacterial numbers.[112] Bifidobacteria have themselves been exploited as a source of enzymes to synthesize NDOs with prebiotic effects.[78,141,142] Metabolic engineering of a β-galactosidase from *Bif. bifidum* substantially reduced hydrolytic activity.[142] The resulting enzyme had highly efficient galactosyltransferase activity and was able to channel carbon to galacto-oligosaccharide synthesis even at relatively low lactose concentrations. Bifidobacteria have also been used to produce synbiotic yogurt through de novo synthesis of galacto-oligosaccharides during milk fermentation.[78] In addition to oligosaccharides, some dietary fibers have been reported to have prebiotic potential.[112,117] These large, slowly fermented carbohydrates may have advantages over oligosaccharides by minimizing rapid gas formation in the gut and promoting fermentation more distally in the colon.

The ability of bifidobacteria to use a wide variety of oligosaccharides and other complex carbohydrates reflects their evolution in the hindgut of humans and animals where the ability to use a wide range of food- and host-derived complex carbohydrates and glycoproteins provides a competitive advantage. Genomic analysis of *Bif. longum* revealed a large number of proteins specialised for the catabolism of carbohydrates.[136] Another consequence of their evolution in an environment with limited availability of simple sugars is that many *Bifidobacterium* strains grow poorly using some monosaccharides, but grow well when supplied with oligosaccharides composed of those same sugars[39,117,143] (Fig. 3). This suggests that bifidobacteria lack transport mechanisms for these simple sugars and import oligosaccharides before hydrolysing and metabolising them.

VIII. HEALTH EFFECTS OF PROBIOTIC BIFIDOBACTERIA

The theoretical benefits of probiotic bifidobacteria include both intestinal and systemic effects, mediated by modulating the functionality of the intestinal microbiota, the gut barrier, and/or the immune system of the host. Both therapeutic and prophylactic roles have been proposed and trailed in animal models and in humans. In the past few years, studies of the probiotic effects of bifidobacteria have been focused in four major areas:

Modulation of the host immune system
Resistance to infectious diseases
Control of inflammatory bowel disease (IBD)
Prevention of colorectal cancer

Although suffering from many limitations in design and control, early studies at least provided indications that the theoretical benefits of consumption of probiotic bifidobacteria

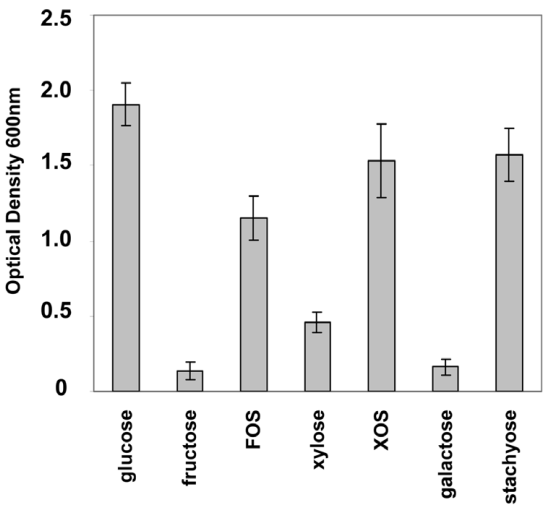

Figure 3 Bifidobacteria are often able to use oligosaccharides as carbon and energy sources more efficiently than the monosaccharides from which the oligosaccharides are composed. This figure displays the growth yield of *Bifidobacterium lactis* following growth using various mono- and oligosaccharides. FOS = fructo-oligosaccharides; XOS = xylo-oligosaccharides. (Modified from Ref. 39.)

could be translated into observable health effects in the host. In recent years, an increasing number of studies have incorporated appropriate attention to the dose, stability, and functionality of the fed probiotic and good clinical design, including randomization of subjects, placebo controls, blinding, crossover of treatments, and meaningful health endpoints. It has now been conclusively demonstrated that some *Bifidobacterium* strains can survive intestinal transit and persist transiently within the colon.[18,25,46,114–116,133,144] Importantly, the strains used as probiotics to date appear to be safe. A few studies have provided measurable improvements in clinical endpoints and a demonstrable amelioration of disease symptoms in the case of atopic eczema in infants.[17,18] Importantly, we are now beginning to gain insights into some of the potential mechanisms of probiotic action by bifidobacteria.

A. Immunomodulation

It is now well documented that probiotic bacteria can modulate both the humoral and cellular immunity of the host.[145,146] Both upregulation of immune protection against pathogens and cancer cells (Tables 2 and 3) and downregulation of aberrant inflammatory responses in allergy and IBD have been observed.[17,147] It is becoming increasingly apparent that modulation of the host immune system may contribute to, or even underpin, many of the health effects attributed to probiotics.

1. Amelioration of Allergy Symptoms

The intestinal mucosal immune system in healthy individuals is able to create a balance between protective immunity against pathogens and tolerance to commensal bacteria and dietary antigens.[148] In atopic food allergy, the balance leading to tolerance is impaired and the immune system responds to the food antigen with an inflammatory response driven by a lymphocyte balance that is skewed towards type 2 T-helper cells (Th2).[119,149,150]

It is now clear that the intestinal microbiota is essential for the development of oral tolerance in the neonate and that the type of bacteria present can influence the immune response and the development of immune homeostasis.[118,119,150–152] The discovery of the importance of the intestinal microbiota in the development of oral tolerance fueled speculation into a possible role for probiotics in the treatment and prevention of atopic disease. Differences have been observed between the composition of the intestinal microbiota in tolerant and atopic infants,[118–125] including a reduced number and adult-like species composition of bifidobacteria in allergic infants.[98,118,119,125]

In perhaps the best demonstrations, so far, of clinically significant ameliorations of disease symptoms by probiotics, supplementations with *Lactobacillus rhamnosus* GG[147] or *Bif. lactis* Bb-12[17,18] proved effective in relieving atopic eczema in infants. The mechanism of action remains speculative and may be different for each strain. Both treatments resulted in deceased markers of inflammation and T-cell activation, but had different influences on serum transforming growth factor (TGF)-β (an anti-inflammatory cytokine) concentrations. Serum TGF-β increased it in the *L. rhamnosus*–treated atopic group, but decreased in the *Bif. lactis* group.[17] Bifidobacteria have been shown to elicit a type 1 T-helper cell (Th1) response, and the observed effect may be due to restoration of a tolerogenic Th1/Th2 balance mediated by interleukin (IL)-12[153] and possibly through induction of IL-10–mediated downregulation of inflammation.[152] *Bifidobacterium* isolates from allergic infants generally failed to induce high levels of

Table 2 In Vivo Studies Demonstrating Stimulation of Innate and Adaptive Immunity by Probiotic Bifidobacteria

Bifidobacterium	Model system	Effect of probiotic	Ref.
Bif. lactis HN019	Humans—healthy elderly. *Bif. lactis* in milk. Milk placebo.	Transient ↑ CD4+, CD25+ and NK cell proportions in blood. Transient ↑ in phagocytic and tumor-cell killing activities.	[21,22]
Bif. lactis HN019	Humans—healthy adults and elderly. *Bif. lactis* in milk with or without oligosaccharides. Milk placebo. Double blind.	Transient ↑ in phagocytic and NK cell activity in PBMCs. Synbiotic effect: the magnitude of the immune stimulation was larger in the group fed *Bif. lactis* + galacto-oligosaccharides.	[20]
Bif. lactis HN019	Humans—healthy elderly. *Bif. lactis* in milk. Milk placebo. Randomised, double blind.	↑ IFN-α from mitogen stimulated PMBCs. ↑ Phagocytic activity of PMBCs	[19]
Bif. lactis HN019	Mice. *Bif. lactis* in milk. Milk placebo.	↑ Phagocytic activity of peritoneal macrophages and blood leucocytes. ↑ Humoral response to oral and injected toxin antigen. ↑ Mitogen-induced T and B cell proliferation. ↑ NK cell cytotoxic activity.	[165]
Bif. bifidum Bb-11	Mice. *Bif. bifidum* in PBS administered orogastrically. Free cells and encapsulated.	↑ Mucosal and systemic immune responses. ↑ Total IgA and IgM synthesis by mesenteric lymph node and Peyer's patch cells and ↑ reactivity of these cells to TGF-β1 and IL-5. ↑ Mucosal total IgA and IgA-secreting cells. Effect enhanced by encapsulation. Immunomodulatory activity was cell-associated and not secreted. No induction of specific *Bif. bifidum* antibody response.	[58]

Table 3 In Vivo Studies Demonstrating Protection Against Infections by Probiotic Bifidobacteria, and Potential Links to Immunological Mechanisms

Bifidobacterium	Pathogen	Model	Effect of probiotic	Ref.
Bif. breve YIT 4064	Influenza. Intranasal challenge following oral vaccination.	Mice. Pre-feeding of autolyzed and heat-treated probiotic prior to challenge. Placebo, orally vaccinated mice without probiotic.	↑ Protection against influenza infection. ↑ IgG to influenza.	[167]
Bif. lactis HN019	*E. coli* 0157:H7	Mice. Probiotic supplementation for 7 days prior to and after oral *E. coli* challenge. Control with no probiotic.	↑ Protection against *E. coli.* ↓ Morbidity and symptoms. ↑ Phagocytic activity and *E.coli*-specific sIgA. ↓ *E. coli* translocation.	[162]
Bif. lactis HN019	*E.coli* and rotavirus	Piglets. Daily oral probiotic supplementation vs. no probiotic control. Naturally acquired infections. Monitoring of *E. coli* and rotavirus shedding.	↓ Diarrhea. ↑ Feed conversion efficiency. ↓ Rotavirus and *E. coli* shedding. ↑ Blood leucocyte phagocytic activity and T cell proliferative response. ↑ Pathogen-specific intestinal antibody levels.	[166]
Bif. lactis HN019	*Salmonella enterica* serovar Typhimurium	Mice. Single and multiple oral pathogen challenge. Low and high pathogen dose. Pre- and post-challenge administration of the probiotic. Non-infected control.	↑ Protection against low and high pathogen dose. ↓ Pathogen translocation to liver and spleen. ↑ Blood leucocyte phagocytic activity and T cell proliferative response. ↑ Pathogen-specific intestinal and systemic antibody levels.	[170]
Bif. lactis Bb-12	Diarrhea in children	Healthy children. Double-blind, placebo controlled. Twice daily dose for 12 weeks.	Indication of reduction in duration of diarrhea. No impact on incidence, which was low in both treatment and control groups.	[18]
Bif. breve YIT 4064	Rotavirus	Infants in an institution. Daily consumption or probiotic for 28 days.	↓ Rotavirus shedding. ↑ Intestinal rotavirus-specific IgA	[164]
Bif. bifidum and *Bif. infantis*	Rotavirus	Mice. Probiotics with or without prebiotics. Control.	↑ Delay in onset, ↓ duration, and ↓ severity of symptoms. No ↑ in effectiveness by prebiotics. ↑ Intestinal and systemic rotavirus-specific IgA.	[163]

IL-10 when used to stimulate macrophages in vitro. In contrast, strains isolated from healthy infants did induce IL-10.[154,155] Interestingly, none of the *Bif. adolescentis* strains, a species usually associated with adult intestinal microbiota but also found in atopic infants,[98,125] induced IL-10.[154,155]

The window of opportunity for intervening in atopy development though probiotic supplementation may be limited to very early childhood when the immune system and microbiota are still maturing. As mentioned previously, the adult microbiota is relatively stable and probiotics may not be capable of significantly impacting the population dynamics to a degree necessary to correct immune deviation in a mature immune system. Limited attempts have been undertaken to test probiotics in adults with milk allergy, but without success.[18] This may have also been due to adult milk hypersensitivity predominantly being driven by mechanisms other than IgE-mediated reactions with Th2-skewed immunity.[156–158] Indications of a clinical benefit in adults with allergic rhinitis have been observed in individuals fed probiotic yogurt containing a *Bifidobacterium* strain and *Lactobacillus acidophilus*. This was linked to an increase in interferon (IFN)-γ secretion by peripheral blood mononuclear cells in the yogurt group, providing indications of an increased Th1 response.[159] The role of the intestinal microbiota in immune development and the use of probiotics for the alleviation of allergy symptoms are now burgeoning areas of research.

2. Stimulation of Innate Immunity

Stimulation of the immune system provides the host with increased ability to fight infections and cancer. The ability of probiotic bacteria to modulate humoral and cellular immunity at the mucosal and systemic level has been established in animal and human studies.[145,148,160,161] Importantly, probiotic lactobacilli and bifidobacteria do not elicit inflammatory responses that could be harmful, but rather transient modifications of immune responsiveness beneficial to the host. Recent in vivo studies of immune stimulation by bifidobacteria are displayed in Table 2. The immune-modulation effects that have been observed for bifidobacteria include:

Increased mucosal IgA production[58,145,162–164]
Stimulation of phagocytic activity of mononuclear cells[19,21,22,165,166]
Stimulation of natural killer (NK) cell activity[20–22,165]
Increased lymphocyte responsiveness to oral and systemic challenge antigen [145,162,167]

In vitro studies have provided further insights into immunomodulation by bifidobacteria. Evidence of strain and species variability in immune effects is demonstrated by the strong differences in cytokine responses they elicit.[154,155] Hence, careful screening and selection of the most appropriate strains to elicit the desired type of immune response for the targeted health effect is essential. The importance of probiotic adhesion to the intestinal mucosa in immune modulation is still poorly understood. Importantly, it appears that adhesion of bifidobacteria to intestinal epithelial cells does not induce undesirable inflammatory responses.[168]

B. Increasing Resistance to Infections

Evidence that probiotics can increase resistance to intestinal pathogens continues to accumulate, with the best protective effects seen to date in human trials for children

with rotavirus diarrhea.[169] The majority of human probiotic studies conducted so far have tested the effects of probiotic lactobacilli. However, a number of recent trials in animal pathogen challenge models and in humans indicate that probiotic bifidobacteria may also afford some protection against enteropathogens (Tables 3 and 4). Several potential mechanisms have been proposed for improved resistance to infections, and those with preliminary evidence from in vitro and animal studies include:

Stimulation of the host immune system[163,166,167,170]
Induction of nonimmune host responses such as increasing intestinal mucus production[138] or reducing toxin receptor expression[171]
Inhibition of pathogens by specific antimicrobials[172–176]
Inhibition of pathogens by metabolic end products[176–178]
Competitive exclusion and inhibition of pathogen mucosal adhesion[176,179–182]
Suppression of pathogen translocation or cell invasion[176,183–185]
Binding or inactivation of pathogen toxins[171]

Recent in vivo studies attempting to link increased resistance to infections to probiotic mechanisms are shown for immune modulation in Table 3 and pathogen inhibition and colonization resistance in Table 4. Human studies investigating the application of probiotic bifidobacteria against rotavirus diarrhea in young children have provided indications that some strains may be protective.[18,164] Results from rodent pathogen challenge models are encouraging, though they are only loose models of human infections. Conclusive evidence of clinically significant protection by bifidobacteria to bacterial pathogens remains to be demonstrated in humans.

C. Maintaining Remission in Inflammatory Bowel Disease

IBD is a group of disorders characterized by chronic, relapsing intestinal inflammation that include Crohn's disease, ulcerative colitis, and pouchitis.[186] A genetic predisposition is recognized, but the etiology remains unknown.[127] The dominant theory, supported by experimental and clinical evidence, is that the inflammation results from a breakdown in normal immune tolerance to components (so far unidentified) of the intestinal microbiota.[127,186] Current steroidal therapies often elicit significant side effects, and since probiotics can modulate both the intestinal immune system and the intestinal microbiota, their application in control of these diseases has been investigated.

A probiotic mixture (VSL#3), which contained three strains of bifidobacteria in addition to lactobacilli and *Streptococcus thermophilus*, was trailed in a randomized, double-blind, placebo-controlled trial of 40 patients with chronic pouchitis.[24] Patients in clinically demonstrated remission were treated with the probiotic or placebo for a period of 9 months and were regularly clinically assessed. Bacteriological analysis demonstrated recovery of the probiotics in feces of the treatment group. The probiotic preparation showed a clear benefit, with only 15% of the probiotic group relapsing compared to 100% in the control group ($p < 0.001$). It is impossible to isolate the role that the bifidobacteria played in this effect. However, a bifidobacteria-fermented milk has now been trailed individually in ulcerative colitis patients with positive effects in a randomized, controlled trial.[26] No adverse effects were observed in these trials indicating that bifidobacteria can be safely used in IBD. It seems likely that selected *Bifidobacterium* strains will prove able to provide clinically significant benefits in the maintenance of remission.

Table 4 In Vivo Studies Demonstrating Protection Against Infections by Probiotic Bifidobacteria, and Potential Links to Pathogen Inhibition or Colonization Resistance

Bifidobacterium	Pathogen	Model	Outcome of probiotic	Ref.
Bif. longum HY8001 (extracellular toxin binding molecule)	*E. coli* 0157:H7 vero cyclotoxin	Mice—oral challenge with *E. coli.* Treatment group dosed intragastrically with *Bif. longum* supernatant before and after challenge. Control—no supernatant.	Inhibition of toxin binding to receptor by direct binding to toxin by a soluble factor produced by the probiotic and reduced expression of the toxin receptor in renal cells of the mice.	[171]
Bif. breve (Yakult)	*Salmonella enterica* serovar Typhimurium	Mice—oral pathogen challenge post colonization with *Bif. breve.* Control—no probiotic. Synbiotic galacto-oligosaccharide.	↓ Pathogen colonization and complete block of translocation. Effect ↑ in synbiotic. Correlation of protection to ↑ organic acid synthesis and ↓ intestinal pH.	[178]
Bif. animalis and *Bif.* spp.Lafti^{TM} B22, B74, B97.	*Salmonella enterica* serovar Typhimurium	Mice—oral pathogen challenge and simultaneous and post challenge administration of probiotics.	↓ Intestinal colonization by pathogen and ↓ symptoms of infection.	[174]
Bif. spp. (CA1 and F9) isolated from infant stools.	*Salmonella enterica* serovar Typhimurium	Germ-free mice. Inoculated with bifidobacteria 1 week before oral pathogen challenge.	↓ Morbidity. Antagonism of *S. enterica* in vitro by lipophilic extracellular molecule(s) <3500Da produced by the bifidobacteria.	[173]
Bif. infantis	Necrotizing enterocolitis (NE)	NE rat model. Probiotic vs. controls of *E. coli* fed and saline fed animals.	Colonization by *Bif. infantis.* ↓ NE incidence and symptom severity. ↑ Survival.	[182]
Bif. bifidum. DVS	*Salmonella enterica* serovar Typhimurium	Conventional and gnotobiotic mice orogastric pathogen challenge. Pre- and post-challenge probiotic administration. Control.	↓ Morbidity and pathology in both models. No difference in intestinal colonization of pathogen between treatment and control groups.	[183]

D. Anticancer Effects

Anticancer effects remain perhaps the most controversial potential health benefit of probiotic bifidobacteria. To date, there is no direct experimental evidence for cancer suppression in humans as a result of probiotic consumption. However, there are indications from in vitro, animal, and human studies that bifidobacteria may contribute to maintaining a colonic environment and host immune system oriented to reduced cancer risk. Possible mechanisms by which probiotics may suppress cancer development are discussed by Reddy[187] and Rafter,[188] and include:

Stimulation of the host's innate immune system
Limitation of genotoxic reactions by the intestinal microbiota
Alteration of physicochemical conditions in the colon
Adsorption or degradation of potential carcinogens
Nourishment of the intestinal epithelium with macro and micronutrients
Production of antitumorigenic or antimutagenic compounds

Stimulation of tumoricidal activity has been observed in ex vivo examination of NK cells from humans fed with probiotic bifidobacteria.[20–22,165] However, direct links between *Bifidobacterium*-induced immune stimulation and cancer suppression in humans are yet to be demonstrated. Reduced fecal mutagenicity has been demonstrated in rodent models and in humans fed bifidobacteria,[189,190] and mutagen binding to *Bifidobacterium* cells has been observed in vitro.[191] A number of studies have investigated the suppressive effects of bifidobacteria on tumorigenesis using carcinogen-induced rodent colon cancer models, with varying results. Some demonstrated strong suppressive effects,[187,190] while others were neutral.[192–194] These variations were possibly due to differences in the *Bifidobacterium* strain and/or the models used. Applying prebiotics in combination with bifidobacteria (synbiotics) appears to enhance anticancer effects in the rodent models.[192,194] Further research is required to link purported mechanisms of probiotic action to cancer-suppressing effects in vivo.

E. Genetically Modified Bifidobacteria in Anticancer Gene Therapy

Though not a probiotic effect, an interesting new application of bifidobacteria is their potential use as targeted vectors to deliver gene therapy to solid hypoxic tumors. The oxygen partial pressure is lower in these tumors than in normal tissues. It has been observed that bifidobacteria injected intravenously in mice selectively germinate and grow in the hypoxic regions of the tumors and not in other tissues.[195] Yazawa et al.[196] demonstrated that a strain of *Bif. longum* injected intravenously into a rodent breast cancer model specifically localized in the tumors. A follow-up study showed that when the strain was genetically modified to synthesize an enzyme able to activate an antitumor drug, the enzyme was expressed only within the tumor.[197] A separate group demonstrated similar results with a *Bif. adolescentis* strain engineered to produce an antiangiogenic drug. When injected intravenously into mice with solid tumors, the bifidobacteria selectively colonized in the tumors, expressed the anticancer drug, and inhibited tumor growth.[198] This represents an exciting new application of bifidobacteria, exploiting these organisms' physiology, safety, and amenability to genetic transformation to provide vectors for targeted gene therapy.

IX. CONCLUSIONS

It is now clear that strains of probiotic bifidobacteria can survive passage through the upper gastrointestinal tract and transiently colonize the colon. Evidence continues to accumulate that certain strains can provide measurable and clinically relevant benefits to human health. The best demonstrations of health benefits thus far reported are for treatment of eczema in allergic infants and for the maintenance of remission in IBD. Some advances have been made towards elucidating possible mechanisms of probiotic action, and modulation of the host immune system is emerging as a key element in many of the beneficial effects seen to date. It is becoming apparent that the age of the host, stability of the microbiota, and maturity of the immune system may have a dramatic influence on some probiotic effects. The role of the microbiota in healthy immune development in infants and possible effects on allergy will be a major focus for research into probiotic bifidobacteria in the short term.

Perhaps lagging in comparison to the pace of advances in other areas of probiotic research are technological solutions to maintaining the stable functionality of environmentally sensitive strains. The application of bifidobacteria in functional foods remains largely limited to fermented dairy products. Technologies are needed to broaden the application of these functional ingredients to a wider range of products, including nonrefrigerated, long shelf-life foods.

Marked progress has been made in our understanding of the population dynamics of bifidobacteria, facilitated by the shift away from culture-based, phenotypic methods towards culture-independent, molecular techniques. However, we are still only at the very beginning of developing an appreciation of the functional relationships between the microbiota and the host in health and disease. An understanding of these relationships is essential to elucidating probiotic mechanisms and is needed to allow scientifically based selection of appropriate probiotic stains. Importantly, uncovering mechanisms of action will add scientific weight to the observed clinical effects. The application of high-throughput functional genomics to the study of host-microbe interactions promises to provide dramatic advances in our understanding of the role, and potential applications, of bifidobacteria in human health. The once theoretical positive impacts on health for probiotics are beginning to be backed by hard scientific data, and with the burgeoning development of new molecular technologies, the pace of scientific research into the roles of bifidobacteria in human health will no doubt accelerate.

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4

The Probiotic Potential of Propionibacteria

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I. INTRODUCTION

Propionic acid bacteria (PAB) are characterized as gram-positive, nonsporing, nonmotile pleomorphic rods. Although some strains may be relatively aerotolerant, they are basically anaerobes that produce propionic acid, acetic acid, and CO_2 as their main fermentation products. Their optimal growth temperature is between 30 and 37°C. PAB were first described by Freudenreich and Jensen in 1906 and given the name *Propionibacterium* by Jensen in 1909.[1] PAB are divided into two principal groups: the classical or dairy PAB and the cutaneous PAB (Table 1). The main habitat of the classical PAB are dairy

Table 1 Two Groups of *Propionibacterium* Species

Classical (dairy) strains	Cutanous strains
P. acidipropionici	*P. acnes*
P. australiense	*P. avidum*
P. cyclohexanicum	*P. granulosum*
P. freudenreichii subsp. *freudenreichii*	*P. lymphophilum*
P. freudenreichii subsp. *shermanii*	*P. propionicus*
P. jensenii	
P. thoenii	
P. microaerophilum	

The species formerly known as *P. inoccuum* has been reclassified as *Propioniferax innocua.*

products, in particular Swiss-type cheeses. However, they have also been isolated from rumen contents, spoiled olives, and spoiled orange juice. The main habitat of the cutaneous PAB is the human skin, where they are part of the normal microbiota. They have also been isolated from the feces of humans, chickens, and pigs. Cutaneaus PAB have occasionally also been isolated from pathological material. Based on 16S rRNA studies, it appears that the classical and dairy PAB are also phylogenetically distinct.[2,3]

II. METABOLISM

The major means of energy generation of PAB is the production of CO_2, propionic acid, and acetic acid through fermentation:

$$3 \text{ glucose} \longrightarrow 4 \text{ propionate} + 2 \text{ acetate} + 2CO_2 \quad (1)$$

This yields 4 ATP per glucose molecule and is thereby more energy efficient than lactic or acetic acid fermentation. The ATP is generated not only through substrate phosphorylation, but also by oxidative phosphorylation.

PAB also have the ability to grow on lactate under anaerobic conditions, a property that is often used for the selective cultivation of PAB:

Classical pathway:

$$3 \text{ lactate} \longrightarrow 2 \text{ propionate} + \text{acetate} + CO_2 + H_2O + \text{ATP} \quad (2)$$

Formation of succinate by CO_2 fixation:

$$3 \text{ lactate} \longrightarrow (2-x) \text{ propionate} + \text{acetate} + (1-x)\ CO_2 + x \text{ succinate} + 3 \text{ ATP} \quad (3)$$

Aspartate as electron acceptor[4]:

$$3 \text{ lactate} + 6 \text{ aspartate} \longrightarrow 3 \text{ acetate} + 3\ CO_2 + 6 \text{ succinate} + 6\ NH_3 + 3 \text{ ATP} \quad (4)$$

For the propionic acid fermentation, biotin and vitamin B_{12} are required. These can be synthesized by the PAB and explains why they are rich sources of these vitamins.

III. PROPIONIC ACID BACTERIA AS DAIRY STARTERS

PAB are commonly used as starter cultures in the dairy industry. Freudenreich and Jensen[5] first described propionibacteria when studying propionic acid fermentaion in Emmental cheese. The species commonly included in dairy products are *P. acidipropionici, P. jensenii, P. thoenii*, and both subspecies of *P. freudenreichii.* PAB play an important role in the production of flavor compounds and the ripening of cheese. Dairy PAB strains are autolytic under the conditions found in cheese and degrade peptides and amino acids present in the cheese. This proteolytic activity leads to an increase in free proline, which contributes to the flavour of PAB-containing cheese.[6] During the maturation of the cheese, the PAB will utilize the lactate that is formed by other starter bacteria present in the cheese.

As outlined above, this will lead to the production of CO_2, which forms the eyes in the cheese.[7] The utilization of lactate will also lead to the formation of propionic acid, one of the typical flavor components of Swiss-type cheeses. Other important flavor compounds include branched-chain fatty acids, which originate from amino acid catabolism.[8]

PAB may also produce exopolysaccharides in milk. This may have important practical implications for modifying the rheological properties of certain fermented dairy products containing PAB or for the production of exopolysaccharide-based thickeners produced by food-grade microbes.[9]

IV. ANTIMICROBIAL ACTIVITY OF PROPIONIC ACID BACTERIA

A. Propionic Acid

Like most other organic acids, propionic acid is inhibitory to certain bacteria and fungi at low pH. Propionic acid bacteria have been added to food in order to function as a preservative. Propionic acid inhibits the growth of microbes through its accumulation within the cell and subsequent inhibition of enzyme activity. Commercial production of propionic acid from the fermentation of sugars by species of PAB has received considerable attention over the years. Milk whey has been suggested as an inexpensive growth medium for the production of propionic acid by *P. acidipropionici* or *P. freudenreichii*.[10] Propionic acid fermentations may be improved through the combination with lactate-producing organisms, e.g. homofermentative *Lactobacillus* species.[11] However, the low concentrations of the acids in the broth and the long incubation time make production and recovery too expensive. Continuous extraction of the acids with solvents has been attempted, but the solvents are in general toxic to the producing cells. Separating the solvent phase from the growth broth by a membrane may eliminate this problem. Alternative extractants like quarternary amines may further improve the removal of propionic acid from broth.[12] It is, however, uncertain if this type of propionic acid production can compete with the petrochemical production through oxidation of liquid phase propane or propanal.

B. Bacteriocins

Bacteriocins have been defined as proteinaceous substances with a narrow inhibitory spectrum, plasmid encoded together with immunity factor.[13] However, many bacteriocins exhibit a rather wide spectrum of inhibition. This makes them of particular interest for potential practical applications, as natural preservatives in foods, or to strengthen the barrier function of the normal intestinal microbiota with bacteriocin-producing probiotics.

Reports on the prevalence of bacteriocin production by PAB are variable. Leversee and Glatz[14] observed that only 4 out of 52 PAB strains tested produced bacteriocins. However, Holo and coworkers[15] observed that the majority of *P. acidipropionici, P. jensenii*, and *P. thoenii* produced bacteriocin-like activity, while only 6 out of 52 tested *P. freudenreichii* exhibited such activity. The prevalence of bacteriocin production may be underestimated when a small number of indicator strains is used. When a large panel of indicator strains was used, almost all strains of PAB exhibited antimicrobial activity against at least one of the tested strains.[16] Despite the apparent prevalence of bacteriocinogenic strains of PAB, few bacteriocins from this genus have been described in detail (Table 2).

Although bacteriocins by definition are proteinaceous and thus are sensitive to proteolytic degradation, it has been observed that mild proteolysis of the bacteriocin produced

Table 2 Bacteriocins Produced by Propionic Acid Bacteria

Bacteriocin	Producing strain	Molecular weight (Da)	Activity against	Ref.
Acnecin	*P. acnes*		Cutaneous propionic acid bacteria	85
	P. freudenreichii subs. *shermanii* ATCC 9616	800–1200	Gram-negative bacteria and yeasts	86
Jenseniin G	*P. jensenii* P126	>12,000	Propionic and lactic acid bacteria	18
Jenseniin P	*P. jensenii* B1264	3000–10,000	Cutaneous propionic acid bacteria, gram-positive and -negative	29
Protease-activated antimicrobial peptide (PAMP)	*P. jensenii* LMG 3032	6383	Propionibacteria, lactobacilli	87
Propionicin PLG-1	*P. thoenii* P127	9328	Yeast, mold, some gram-negative, lactic acid bacteria	14
Propionicin T1	*P. thoenii* 419 *P. thoenii* LMG 2792	7130	Propionic acid bacteria	88
Propionicin SM1	*P. jensenii* DF1	22,300	Yeast, mold, lactic and propionic acid bacteria	89
Propionicin SM2	*P. jensenii* DF1	13,600		16

by *P. jensenii* B1264 increases its inhibitory activity and its inhibitory spectrum.[17] This may provide a new type of antimicrobial activity, where activation takes place first in an environment with proteolytic activity, e.g., the gastrointestinal tract.

The potential application of bacteriocins or bacteriocinogenic strains is great. However, in general the formation of bacteriocins by PAB is slow. It may require 3 days for propionicin T1 or 7 days or more for jenseniin G and propionicin PLG-1. Even after this long incubation period, the concentration of bacteriocins may be very low and antimicrobial activity needs to be concentrated in order to be detectable.[18] This may relate to the fact that production in liquid media is in general poor compared to solid media and culture conditions need to be very carefully optimized to obtain higher yields.[19] These difficulties in the production are likely to limit the possibilities for practical applications of the bacteriocins of PAB. To what extent bacteriocins are produced in situ by PAB in a food matrix or in the intestine is currently unknown.

C. Antiviral Activity

PAB have also been observed to produce antiviral substances; propionins. These substances were found in the cell-free extracts of *P. freudenreichii* and suggested to be of proteinaceous nature[20] with a molecular weight of 1000 to 1500 Da.[21] However, since these early reports, no further investigations have been reported on this subject.

D. Applications of PAB as Preservatives

Propionate is commonly used in the manufacture of bread to inhibit spoilage organisms. It is a much more powerful inhibitor of yeasts and molds than, e.g., acetate or lactate.[22] Propionic acid (E280) and sodium, potassium, and calcium propionate (E281, E282, E283, respectively) are therefore allowed as preservatives in most countries in the world. The use of propionates will retard mold growth by several days, thereby extending the shelf life. Propionic acid bacteria are not normally found in sourdough breads,[23] but may be added to aid in the inhibition of spoilage organisms.

Two products on the market exploit PAB and/or their metabolites for preservation purposes. Microgard™ is a skim milk product pasteurized after fermentation with *P. freudenreichii* subsp. *shermanii.* It is used to preserve dairy products such as cottage cheese and inhibits the growth of some molds and gram-negative bacteria, but not gram-positive bacteria.[24] The antimicrobial activity of Microgard is attributed to a 700 Da heat-stable peptide and, to a lesser extent, to the presence of propionic acid and acetic acid. Al-Zoreky and coworkers claimed that the inhibitory activity can be increased by varying the fermentation conditions under which Microgard is produced, but did not provide any details.[25] Microgard is mainly marketed in the United States and Canada. The other commercial product using PAB for preservation is Bioprofit™, which is mainly used in Europe. Bioprofit consists of a combination of *P. freudenreichii* subsp. *shermanii* JS and *Lactobacillus rhamnosus* LC-705. It is effective in suppressing the outgrowth of spoilage organisms in dairy products and sourdough bread. The combined effect of the two strains against spoilage yeast, molds, and *Bacillus* ssp. was stronger than the effect of either culture alone. This was suggested to be due to a synergistic effect of the different metabolites of the culture combination. The active components in Bioprofit, other than acetic, lactic, and propionic acids, are currently not known.[26]

PAB have also been suggested for the preservation of meat products. Such applications relied, however, solely on the production of propionic acid to replace chemically manufactured propionate.[27]

PAB have been successfully applied in silage to prevent the growth of aerobic bacteria or yeasts and molds. The addition of PAB prolongs the storage life of the silage through the production of propionic acid and by lowering the pH. The silage also has a better aerobic stability after opening. This results in a better quality silage with a higher nutritional value, which may benefit animal husbandry. In addition to the use of pure cultures of PAB, combinations with lactobacilli, such as Bioprofit, have been observed to produce good quality silages.[26] However, combinations of lactobacilli and PAB have also been observed to perform less then either strain alone, probably through antagonistic acitivities.[28] This clearly indicates that strain combinations should be carefully assessed.

An interesting potential application of PAB bacteriocins has been suggested by.[29] *P. jensenii* B1264 produces a bacteriocin (Jenseniin P) (see Table 2) that is active against, among others, *P. acnes* and could thus be used in the treatment of acne vulgaris. Whether this actually will work in vivo remains, to be determined.

V. PROPIONIC ACID BACTERIA AS PROBIOTICS

The literature concerning the potential probiotic properties of PAB is very limited compared to that about lactobacilli and bifidobacteria. However, in recent years an increasing number of reports on the potential health benefits of PAB have been published.

It is still uncertain if PAB represent an important fraction of the intestinal microflora. Some reports describe the detection of relatively high levels of mainly cutaneous PAB,[30] while others report low or nondetectable levels of PAB.[31–33] Although potential probiotics often originate from the intestine, in the case of PAB it may be advisable not to use intestinal isolates, as these usually belong to the group of cutaneous PAB. This group is associated with disease (see below) and is therefore not appropriate. However, the classical PAB have a number of properties that make them good probiotic candidates.

Survival of gastrointestinal transit is an important selection criterion for probiotics. Several PAB strains have been observed to survive exposure to low pH, which simulates passage through the stomach.[34,35] Although incubation at low pH can affect the viability and the enzyme activity of PAB to a varying extent, this can be counteracted through the presence of milk or cheese, indicating that PAB present in a food matrix may exhibit better survival.[36] The ability of PAB to survive low pH can also be significantly improved by a short exposure to a nonlethal pH (e.g., pH 5). Even exposure to other nonlethal stresses may provide protection against low pH.[37] How this preadaptation can be maintained in a product to facilitate better survival of gastric transit remains to be determined. Strains of PAB have also been observed to resist exposure to bile salts.[35] Despite their resistance to bile, some strains were apparently permeabilized by it, allowing more lactose to enter the cell, which was subsequently hydrolyzed. Exposure to bile also stimulated the production of β-galactosidase, further increasing the activity. This can be expected to contribute to a relief of lactose intolerance symptoms. Bile-sensitive strains of PAB were also permeabilized, but their β-galactosidase activity decreased during prolonged exposure to bile,[38] and their contribution to lactose digestion may therefore be more limited. PAB have been observed to survive gastrointestinal transit in relatively high levels,[39] but were not observed to persist in the human intestine after feeding was stopped.[32] Mice fed *P. acidipropionici* CRL1198 were found to excrete PAB at levels of 10^8 colony-forming units (CFU)/g one week after cessation of PAB feeding.[40]

Another important selection criterion for probiotics is their ability to adhere to the intestinal mucosa. This may allow a probiotic organism to persist longer in the gastrointestinal tract to contribute to the competitive exclusion of pathogens and to modulate the immune response.[41] Some PAB have been assessed for their ability to adhere to intestinal mucosal models. It was observed that *P. freudenreichii* ssp. *shermanii* JS adhered to Caco-2 enterocyte-like tissue culture cells at levels similar to *L. rhamnosus* GG, a probiotic strain known to adhere well to the intestinal mucusoa and intestinal mucosal models.[42] This adhesion was reduced by 24% through prior adhesion of *L. rhamnosus* LC-705, indicating a competition for binding sites. Adhesion of four PAB to human intestinal mucus was observed to be low and mainly due to nonspecific interactions with the mucus.[41] Interestingly, this adhesion could be increased two- to threefold through prior adhesion of other probiotics, such as *L. rhamnosus* GG or *B. lactis* Bb12. These observations suggest that certain combinations of PAB and other probiotics may be synergistic, while others may be antagonistic. The skin has been suggested as a novel area for probiotic use of PAB. Classical PAB are nonpathogenic and would be well suited to compete with the cutaneous PAB and other skin microbes. In addition, some strains of classical PAB have been observed to adhere well to keratin, the main protein in skin.[43] Although in vitro adhesion assays are useful screening tools, the results need to be confirmed in vivo. Good correlation between in vitro adhesion and adhesion in mice for six strains of PAB has been observed.[44] Strain *P. acidipropionici* CRL1198, which earlier had been observed to persist for at least a week, exhibited the highest adhesion both in vitro and in vivo.

Thus, PAB appear to fulfill the major selection criteria for probiotics and may therefore have a potential to be used as such. However, fulfilling probiotic selection criteria does not guarantee probiotic efficacy. This will have to be assessed in vivo in the target host.

A. Stimulation of *Bifidobacterium* Growth

Organic acids, and in particular propionic acid, have been observed to stimulate the growth of bifidobacteria.[45] Propionate is therefore also often added to media for the selective enumeration of bifidobacteria.[46]

The ability of PAB to stimulate the growth of bifidobacteria was first observed by Kaneko and co-workers.[45] They observed that PAB and several intestinal bacteria, such as *Bacteroides, Enterobacter*, and *Enterococcus*, released growth-stimulating factors for bifidobacteria in the growth medium. The stimulatory activity was purified and identified as 2-amino-3-carboxy-1,4-naphthoquinone (MW 217 Da).[47] Other workers have observed that PAB stimulation of the growth of bifidobacteria appears to be a relatively common phenomenon. Warminska-Radyko and coworkers[35] observed that all 27 PAB tested stimulated the growth of at least some bifidobacteria. However, 15 strains of PAB were observed to inhibit the growth of some bifidobacteria. It is not certain if these bifidogenic properties of selected PAB strains are expressed in vivo. Bouglé and coworkers[32] observed that feeding 5×10^{10} CFU of *P. freudenreichii* SI26 and SI 41 daily increased the fecal *Bifidobacterium* one week after stopping the consumption of PAB. At that time, no PAB were detected in the feces, and it is therefore unlikely that this increase is due to the previously consumed PAB. However, an increase in the level of fecal bifidobacteria in children upon consumption of *P. freudenreichii* ssp. *shermanii* has been observed.[48] Although bifidobacteria are considered to be the main beneficial members of the intestinal microflora, a mere increase in their numbers does not necessarily contribute to a beneficial health effect. The specific health benefits of increased fecal bifidobacteria levels will need to be determined.

B. Stimulation of Propionic Acid Bacteria Growth by Lactobacilli

Selected lactobacilli have been observed to stimulate the growth of propionic acid bacteria. This may be due to the production of lactic acid from hexoses that cannot be utilized by PAB. The produced lactic acid will subsequently serve as a carbon and energy source for the PAB. However, other mechanisms may also be involved. *L. helveticus* DPC 4571 stimulated the growth of *P. freudenreichii* DPC 3801 in milk. This appeared to be due to the production of tetra-, penta-, and hexa-peptides from casein, which are produced by cell wall–associated proteinases of *L. helveticus* DPC 4571. Also, peptides from casein produced by other *L. helveticus* strains were found to be growth stimulating.[49] The health implications of this are uncertain. However, casein-derived peptides are apparently able to survive digestion since selected peptides retain their ability to lower blood pressure upon consumption.[50] This could indicate that dairy products that contain growth-promoting peptides for PAB will retain their functionality and may be able to stimulate PAB growth in the intestine, although this would need to be assessed in vivo.

C. Changes in Intestinal Microflora Composition

Feeding of PAB to mice has been observed to cause changes in the composition of the fecal microflora, such as a reduction in the level of coliforms.[51] Sidorchuk and

Bondarenko[48] observed an increase in fecal bifidobacteria and lactobacilli and a decrease in enterobacteria and staphylococci in children with microflora abnormalities. Whether this will also contribute to a protection against pathogens remains to be determined.

D. Fecal Enzyme Activity

The intestinal microflora is able to generate mutagens, carcinogens, and tumor promoters from dietary and endogenously produced precursors. Bacterial enzymes involved in the production of these harmful substances are azoreductase, nitroreductase, nitrate reductase, β-glucuronidase, and β-glycosidase. The ability of certain probiotics to reduce the level of these enzymes in feces is considered to be a desirable trait. It was observed that *P. acidipropionici* CRL1198 reduced the fecal β-glucuronidase in mice.[51] The activity of other fecal enzymes was not changed upon feeding either of the five tested PAB. Consumption of a combination of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC-705 was found to cause a reduction in the level of fecal azoreductase in elderly subjects.[52]

In addition to altering the fecal enzyme activity, *P. freudenreichii* ssp. *shermanii* JS, in combination with *L. rhamnosus* LC-705 has also been observed to be able to reduce the fecal level of the carcinogen aflatoxin B1 in volunteers.[53] Although the observations that PAB can reduce fecal enzyme activity and remove aflatoxins are promising, it remains to be determined whether this will actually lead to a reduced risk for cancer in humans.

E. Cholesterol-Lowering Effects

P. acidipropionici has been shown to reduce serum cholesterol levels in mice.[40] The mechanism by which this occurred is not known, though it has been hypothesized to relate to the absorption of cholesterol to the bacteria whereby the cholesterol would be excreted from the body without being reabsorbed. In addition to absorption or metabolism of cholesterol, the production of propionic acid may contribute to the lowering of serum cholesterol. Acetate, which is produced by bacterial fermentation, has been observed to cause an increase in serum cholesterol, which could be counteracted by the presence of propionate.[54] PAB, which are main propionate producers, may in that way contribute to lowering of serum cholesterol.

The reduction of serum cholesterol levels has been assessed for many probiotics, with conflicting results. It therefore remains to be determined whether PAB will perform better.

F. Production of Conjugated Linoleic Acid and Other Biologically Active Components

Conjugated linoleic acid (CLA) has attracted extensive interest because of its potential beneficial physiological effects and anticarcinogenic and antiatherosclerotic properties.[55–57] CLA consists of a mixture of isomers of octadecadienoic acids with conjugated double bond of which the *cis-9,trans-11* configuration is considered to have highest biological activity.[56] Because CLA is formed in the rumen by microbes such as *Butyrovibrio fibrisolvens* through conversion of linoleic acid (LA), dairy products and beef are the main source. However, PAB have been observed to be able to form CLA from LA. In a screening it was observed that three out of the six tested PAB were able to form CLA, while none of the tested lactobacilli or lactococci were able to do so.[58] CLA is mainly produced by growing cells, but nongrowing cells are also able to convert LA to CLA. Growing cells

may accumulate 80–87% CLA of the initial LA present in the culture. It has been suggested that the isomerization is a detoxification reaction for LA.[58,59] The practical implications of this remain to be determined. It is not likely that PAB will produce appreciable amounts of CLA in situ in the human intestine due to low local levels of LA. Nor is it certain whether the biological production of CLA will be able to compete with the chemical production, although the former will produce a larger fraction of the biologically important *cis*-9, *trans*-11 isomer. Feasible applications may be in the field of CLA-enriched fermented dairy products. Some dairy products are richer in CLA then others, depending on the microbiota present.[56] Inclusion of PAB may enrich these products with CLA, although their inclusion should not affect the taste of the product negatively. In addition, it has to be determined if such CLA-enriched products indeed have additional health benefit over the traditional products.

In the formation of propionic acid, PAB use enzymes that contain several specific cofactors such as vitamin B_{12}, folic acid, and biotin. PAB have therefore long been known to be efficient producers of these vitamins and are used to commercially produce vitamin B_{12}. The production of vitamin B_{12} is a two-stage process involving anaerobic growth followed by aerobic incubation in the latter phase of the production.[60] To further increase the yield of the vitamin, the precursor dimethylbenzimidazole can be added to the fermentation mixture.[61]

Although green vegetables are the main dietary source of folic acid, milk does contain reasonable amounts of folic acid (50–100 μg/L). Because some dairy starters produce folic acid, certain fermented dairy products like Brie and Camembert cheeses may contain 10 times higher levels of folate than milk.[62] It can be expected that PAB-containing cheeses are good sources of folate.

PAB use biotin as a cofactor in their propionic acid synthesis. It is, however, not known how much their biotin content contributes to the dietary intake of this vitamin from foods containing PAB. Another potential nutritional contribution of PAB is their mineral content. PAB have been found to contain relatively high amounts of Mg, Mn, and Co.[63] However, since the biomass of PAB that is consumed is in general low, it remains to be determined whether this represents a significant source of these minerals.

In response to external stresses, PAB can accumulate varying amounts of trehalose. Trehalose is a low-energy sugar because it is only partially digested in the human gastrointestinal tract and is poorly metabolized by many microbes. It can therefore be used as a dietetic sugar.[61] Trehalose has been observed to be utilized by commensal *Escherichia coli* but not by enterohemorrhagic *E. coli* O157:H7. This leads to a displacement of the latter by the commensal *E. coli.* Indeed, the feeding trehalose has been observed to reduce the carrier level of *E. coli* O157:H7 in ruminants. This may provide an additional control method for this pathogen.[64] Because PAB are normal members of the rumen microflora,[65] it could be hypothesized that trehalose-excreting PAB would have a similar effect.

G. Immunomodulation

Cutaneous PAB have been observed to modulate the immune response upon injection of whole cells or cell fragments in animals. Tsuchida and coworkers[66] observed that injection of *P. acnes* into mice activated macrophages and significantly reduced systemic metastasis formation after tumor transplantation. Similarly, Pulverer and coworkers[67] showed that injection of *P. avidum* into mice reduced the number of liver tumors induced

by RAW 117-H110 lymphosarcoma. Interestingly, similar results have also been obtained in human studies. Preoperative injection with *P. granulosum* KP-45 in patients with colorectal carcinoma resulted in a reduced number of postoperative wound infections and significantly increased the survival of patients treated for stage I and stage II colorectal cancer. The recurrence rate was significantly less in the treatment group as compared to the control group after 76 months.[68]

Simultaneous administration of *P. acnes* and Brucella vaccine has been shown to improve the response to the vaccine, both through activation of macrophages and through T-cell–mediated cytolytic activity.[69] Injection of *P. granulosum* or its cell walls into mice prior to infection with herpes simplex, vaccinia, or mouse hepatitis virus led to a significant reduction in mortality among the treated mice. Treatment of the mice with *P. granulosum* on the same day as the virus infection did not always result in a protective response.[70]

Thus, injection of selected PAB appears to result in a potentially beneficial modulation of the immune response in animals and in humans. There is some evidence that oral administration of PAB may provide an immunomodulatory response. Oral administration of *P. acidipropionici* CRL 1198 was observed to increase the phagocytic activity of macrophages[40] in mice. Oral administration of *P. freudenreichiii* ssp. *shermanii* JS to mice stimulated the proliferative activity of B and T lymphocytes.[71] It can be hypothesized that oral administration of PAB may induce apoptosis in colorectal carcinoma cells via the production of short-chain fatty acids, in particular acetate and propionate.[72] In humans, immune modulation after oral administration of PAB has been observed. *P. avidum* KP-40 was found to counteract the immune depression caused by intensive sport activities and normalized lymphocyte counts and activities.[73]

The main immunomodulatory activity has been obtained through injection of cutaneous PAB. This is, however, not the common method of application for probiotics and may provide a safety concern for applications in human medicine (see below). It is therefore important that research on the immune-modulating effects of PAB be more focused on oral administration and the use of classical PAB instead of cutaneous PAB. This will provide safer products for assessment in humans.

H. Protease Activity

Dairy propionibacteria are known to possess membrane-bound proteases that are able to hydrolyze casein.[74] Proline iminopeptidases have also been isolated from PAB; the enzymes are released when the cells undergo autolysis during the ripening of the cheese.[6]

PAB have been suggested to be the predominant proteolytic bacteria found in human feces. Extracellular proteases were found to be responsible for the activity. Although the proteolytic activity was in general low, the authors concluded that PAB may make a significant contribution to proteolytic activity in the colon due to the high levels of PAB observed.[30] However, as stated above, not all investigations have reported high levels of PAB in human feces. The general contribution of PAB to protein digestion therefore remains to be determined. In addition, it is not certain whether proteolytic activity in the colon is a desirable trait. Proteolysis will yield free amino acids, which can be decarboxylated to biogenic amines or give other substances that are potentially harmful to the host when absorbed from the colonic contents.[75] In particular, the cutaneous PAB have been observed to be able to produce inflammatory substances such as tryptamine and

histamine.[76] The health consequences of in vivo proteolytic activity of PAB need therefore to be carefully examined.

VI. SAFETY

No data are currently available concerning the possible acute or chronic toxicity of PAB. Although dairy PAB are consumed in relatively large amounts in cheese (10^8 CFU/g) without any known ill effects, the use of PAB as probiotics would involve the consumption of larger numbers. However, *P. freudenreichii* ssp. *shermanii* T-73 was not observed to cause any ill effects on the general state of health of mice, guinea pigs, or rabbits upon intraperitoneal administration of 10^9–10^{10} CFU. Nor did it exhibit any cytotoxic effects on tissue culture cells.[48] Nevertheless, it would be advised that toxicity studies be performed prior to widespread use of PAB as probiotics, even though it seems unlikely that any serious risk exists.

Unlike dairy PAB, cutaneous PAB have been linked to different diseases. *P. acnes* may be involved in development of acne vulgaris, although it probably dues not initiate it.[77] *P. acnes* has also been found to be associated with gallstones. It is, however, unclear whether the organism is directly involved in the etiology of gallstone formation.[78] Cutaneous PAB and in particular *P. acnes* may cause opportunistic infections in patients after surgery and implantation of foreign bodies. Other predisposing factors include diabetes, immune deficiency, malignancy, or trauma.[79,80] Treatment involves intravenous antibiotic therapy and removal of the infected tissue/foreign body. A succes rate of 80% has been observed.[79] Cutaneous PAB have also been tentatively linked to rheumatoid arthritis.[81] This could relate to the strong nonspecific immune modulation exerted by many PAB, as mentioned above. PAB have also been connected to bacteremia in association with endodontic therapy[82] and meningitis in children, where ear, nose, and throat infections have been indicated to be the main predisposing factors.[83] Infections in which PAB have been reported to be involved in the etiology have without exception been caused by cutaneous PAB. Members of this group are known to carry virulence factors such as hyaluronidase activity.

P. acnes is also responsible for the breakdown of skin triglycerides, giving rise to a large number of both long-chain and short-chain free fatty acids. In addition to having an unpleasant smell, they also function as important signaling substances for mosquitos[84] and may thus indirectly contribute to the development of disease spread through mosquitos.

Because of the potential link between cutaneous PAB and infections, strains belonging to this group do not appear to be particularly suited as probiotics. Dairy strains have so far not been found to be associated with disease[79] and appear to be better candidates for probiotic use. Nevertheless, safety assessments should be considered even for strains from this group.

VII. CONCLUSIONS

The above overview suggests that selected PAB strains have a good potential for use as probiotics. In particular, members of the group of dairy PAB may offer a range of organisms that could be beneficial to the health of the consumer and can be considered to be safe due to their long history of safe use in fermented dairy products. Although mainly cutaneous PAB have been shown to modulate the immune response, some dairy PAB

have been shown to have immunomodulatory activity, even by oral administration. This is encouraging because the use of cutaneous PAB is less desirable as they have been associated with disease and elaborate safety investigations will be needed to guarantee their safety and include them in novel functional foods.

A number of important issues remain to be resolved. To what extent are PAB members of the normal intestinal microbiota and what is their ecological function? The proposed health effects discussed above need to be verified in well-conducted clinical studies. Finally, because PAB produce a rather strong flavor, it will be a challenge to produce functional foods, other than cheese, containing PAB. Thus, there is considerable potential for the probiotic use of PAB, but much work remains to be done.

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5

Industrial Use and Production of Lactic Acid Bacteria

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I. INTRODUCTION

Since the early 1900s there has been a marked worldwide increase in the industrial production of cheeses and fermented milks. Process technology has progressed toward increasing mechanization, increasing factory sizes, shortening processing times, and processing larger quantities of milk. Fermented milk can be used in more than 1000 products for and affects flavor, texture, and final product quality.

All this is reflected in enormous demands for starter cultures, their activity, stable quality, and bacteriophage resistance. The art of making cultured food products by using the former day's whey or fermented product for today's process has been changed to a science with exact knowledge of the factors influencing the specific starter species and strains.

Many outstanding reviews have been published about the metabolism, physiology, genetics, production, and use of starter cultures. Thus, in this chapter the general view of the most important factors concerning starter activity, its effect on the final product, and industrial starter production are discussed. The discussion of industrial production of starters is mostly based on the author's experience.

II. MESOPHILIC AND THERMOPHILIC STARTERS USED BY THE DAIRY INDUSTRY

The starters used in dairy products can be divided into mesophilic and thermophilic starters according to optimum growth temperature. Mesophilic cultures grow in temperatures of 10–40°C, with the optimum around 30°C. Thermophilic starter cultures have their optimum growth temperature between 40°C and 50°C.

Mesophilic starter cultures, composed of acid-forming lactococci and often of flavor producers, are used in the production of many cheese varieties, fermented milk products, and ripened cream butter.[1] Thermophilic starters are used for yogurt and for cheese varieties with high cooking temperatures (Emmental, Gruyère, Comte, Grana).

The starter cultures are usually composed of different species or of several strains of a single species. Differently composed starters can be categorized as follows:

Single-strain starter: one strain of a certain species
Multiple-strain starter (defined strain starter): different known strains of one species
Multiple-mixed-strain starter: different defined strains of different species
Raw mixed-strain starter: species and strains partly or all unknown

The traditional raw mixed-strain starters are widely used, especially in fermented milk products and ripened cream butter. All the categories are known as cheese starters, and the trend is toward multiple-strain starters. Mesophilic cultures are either raw mixed-strain or multiple-strain cultures in Europe and North America. The multiple strains are used singly, in pairs, or in multiples, and their use was pioneered in New Zealand by Whitehead[2] to avoid open-texture defects in cheddar cheese caused by flavor-producing organisms in mixed cultures. The defined strain cultures have led to the understanding of strain interaction with phages.[3] During the 1970s the big cheese plants started to use defined phage-insensitive strain systems with good success.[4–6] Since this development, the multiple-strain starters have become popular in Australia,[7] the United States;[8–10] and Ireland.[11] About 85% of all cheddar cheese was produced with these starters in 1985.[12]

In the Netherlands a totally different starter system is applied based on the difference in phage sensitivity between the starters propagated in the laboratory ("L cultures") and in practice ("P cultures"). Under aseptic conditions the L cultures became dominated by phage-sensitive strains, while P cultures propagated in nonaseptic dairy conditions attained the optimum balance between phage-sensitive and -insensitive strains. The cultures used are P cultures kept in The Netherlands Dairy Research Institute and distributed to the cheese factories for large-scale production.[13]

Thermophilic cheese cultures can also be divided into two categories: raw mixed starters and defined strain starters with multiple or single strains. Raw mixed cultures are widely used in traditional cheese making in Switzerland, France, and Italy. The mixed cultures contain *Streptococcus thermophilus* and different species of *Lactobacillus*: *L. helveticus*, *L. lactis*, *L. bulgaris*, *L. fermentum*, and *L. acidophilus*. The raw mixed cultures can be natural whey cultures or produced by macerating dried calf vells in the previous day's cheese whey. In spite of the use of these unknown mixed cultures, the need to know the exact properties of starter strains is well recognized in Switzerland. During the 1970s defects in secondary fermentation occurred in Emmental cheese, caused by starters that were too proteolytic, which stimulated propionic acid fermentation and production of CO_2.[14] After research on this defect the variety of starter cultures decreased, and they are distributed weekly to cheese factories by the Research Institute.[15]

Single-strain starters containing *Str. thermophilus* and *L. helveticus* have been developed in France[16] and used for Gruyère and Emmental cheese as frozen concentrated cultures for bulk starter or direct inoculation of cheese milk. Single-strain starters have been used in Finland for Emmental cheese, starting in the 1930s with *Str. thermophilus* strains; by the 1950s single strains of *L. helveticus* and propionic acid bacteria were also used. Because of the decrease of natural flora in raw milk during the 1970s and 1980s, more species of lactobacilli and propionic acid bacteria were needed for Emmental cheese to accelerate the ripening time and to improve the taste and eye appeal.

A. Mesophilic Species and Types of Starters

The species composition of most mesophilic starters include *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* var. *diacetylactis*, *Leuconostoc lactis*, and *Leuconostoc cremoris. Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* are acid-producing microorganisms. When starter cultures contain only these species, they are characterized as O type. *Lc. lactis* ssp. *lactis* var. *diacetylactis* and *Leuconostoc* sp. are citric acid–fermenting bacteria. When the only citrate-fermenting species present is *Lc. lactis* ssp. *lactis* var. *diacetylactis*, the culture is of D type. When the *Leuconostoc* sp. is the only aroma producer, the culture is of B (or L) type. When both aroma-forming species are in the culture, it is called BD (or LD) type.

Several combinations of single or multiple strains of lactic acid bacteria are currently used in cheese making. The various starter systems used in the dairy industry are either mixed cultures in which the composition of the mixture is not defined or cultures containing well-defined single or multiple strains.

1. Dutch: Mixed cultures, coming from dairies or butter plants, are propagated, without isolation, in order to keep a composition as close as possible to the original culture. When those cultures are propagated under aseptic conditions, very few bacteriophage attacks are unnoticed.
2. New Zealand: This system is used in many Anglo-Saxon countries and utilizes multiple-strain cultures composed of a small number of defined strains. Either the same culture containing two to six strains is used alone for a long time, or several cultures are used in rotation in order to prevent bacteriophage attacks. In the latter case, the cultures have to have different bacteriophage sensitivity profiles.
3. Australian: This system consists of a single strain, replaced as soon as possible in case of a bacteriophage attack. From the sensitive strain screened in the factory, a secondary resistant strain is derived to replace the original strain.

A combination of these two last systems has been successfully used in the United States and Ireland by selecting secondary resistant strains and including them afterwards in multiple-strain cultures.

B. Thermophilic Species and Types of Starters

Thermophilic organisms belong to two genera: *Lactobacillus* and *Streptococcus. Lactobacillus* is a large genus with over 50 homo- and heterofermentative species. Only a few of these are involved in milk fermentations. *Lb. delbrueckii* ssp. *lactis* and

Lb. helveticus are the starter lactobacilli for cheeses with high cooking temperatures, and *Lb. delbrueckii* ssp. *bulgaricus* (formerly known as *Lb. bulgaricus*) is a component in yogurt together with *Str. salivarius* ssp. *thermophilus. Lb. acidophilus* is of intestinal origin and is widely used in different kinds of milk products because of its believed beneficial effects on human and animal health.

Lactobacilli are used in combination with *Str. thermophilus*. This combination is naturally selected because of the high temperatures used in the fermentation of certain cheeses and yogurt. *Lb. lactis*, *Lb. bulgaricus*, and *Str. thermophilus* do not metabolize galactose, and thus lactose metabolism by *Str. thermophilus* results in galactose accumulation in the medium (Thomas and Crow, 1984). For this reason only galactose-fermenting lactobacilli should be used as starters together with *Str. thermophilus*.[17]

A symbiotic relationship exists between *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus. Lb. bulgaricus* stimulates *S. thermophilus* by releasing amino acids, while the latter produces formic acid–like compounds, which promote the growth of *Lb. bulgaricus*. Many outstanding reviews have been written in this area describing the symbiosis in detail.[18–21] Some data have been published about the symbiotic relationship existing between *Str. thermophilus* and *Lb. helveticus*, but the actual compounds involved are not known yet.[22]

The lactobacilli with lower optimum growth temperatures, *Lb. casei* and *Lb. plantarum*, grow in cheese as natural contaminants. Some strains of *Lb. casei* produce diacetyl from citrate, but this species is used as a starter only by the Japanese in making fermented milk, yakult.

C. Starter Function

Among the physiological functions of lactococci are several of great importance in cheese manufacturing and maturation, influencing the final organoleptic qualities of the cheese:

Fermentation of sugars, leading to a pH decrease important in the clotting phenomenon and reduction or prevention of the growth of adventitious micro-flora
Protein hydrolysis which causes the texture and, partially, taste of cheese
Synthesis of flavor compounds
Synthesis of texturing agents, which may influence the consistency of the product
Production of inhibitory components

Since the lipolytic activity of lactococci is very low, it has no major influence on the technology and, consequently, is not further treated here.

1. Acid Production

Lactose is the major fermentable sugar found in milk at levels of 40–50 g/L. The glucose moiety of lactose is used faster than the galactose moiety by lactococci. At the end of the growth phase, less than 0.5% of the lactose is used by lactococci.[23] The fermentation product of lactococci is L(+)-lactic acid.

Sugar Transport Across the Cell Membrane. The bacterial transport of lactose, glucose, and galactose across the cytoplasmic membrane has been well characterized. Two different mechanisms have been found: the permease and the phosphoenol

pyruvate-phosphotransferase (PEP/PTS) systems. The permease system is found in thermophilic species and the leuconostocs, and the PEP/PTS system in the lactococci. Lactose is transported via PTS. This system, composed of two enzymes and a soluble factor together with a thermoresistant protein, is PEP-dependent. In the PEP/PTS system lactose is transported into the cell via a complex system by which lactose is phosphorylated to lactose phosphate and thus transported across the cell wall. The lactose phosphate is hydrolyzed to glucose and galactose-6-phosphate by phospho-β-galactosidase (p-β-gal). Lactose can also be transported by a permease system. This system, which requires energy, uses the ATP of the cell. Inside the cell, lactose is hydrolyzed to glucose and galactose by β-galactosidase (β-gal). There are relatively few studies on the transport of lactose by thermophilic cultures. Contradictory results have been published about the transport of lactose in *Str. thermophilus.* Both permease and PEP/PTS systems [24] and only permease [25] have been suggested. Lactobacilli contain more β-gal than p-β-gal,[26] implying that permease is the most important transport system.

Carbohydrates Catabolism. After transportation, the sugars can be either lactose phosphate, glucose phosphate, galactose phosphate, or corresponding free sugars. These molecules can be metabolized by three different pathways. The lactose phosphate is hydrolyzed by a β-gal to give glucose and galactose-6-phosphate. Then, the glucose moiety is catabolized through the glycolysis pathway (Embden-Meyerhof-Parnas pathway), while galactose-6-phosphate is metabolized along the D-tagatose-6-phosphate pathway. Galactose is used in the Leloir pathway. Carbohydrate metabolism is controlled by both repression and retroinhibition. Repression is a mechanism that controls enzyme synthesis, and retroinhibition controls enzyme activity.

2. Proteolytic Activity

All starter culture species are nutritionally fastidious, requiring many amino acids and growth factors for adequate growth. Lactic acid bacteria are only mildly proteolytic compared to, e.g., *Bacillus* and *Pseudomonas*. Lactic acid bacteria utilize the polypeptides generated by milclotting enzymes and by bacterial cell wall proteinases and therefore are responsible for the casein degradation. The combined action of proteinases and peptidases provides the cells with peptides and free amino acids. Then peptides and amino acids are transported across the membrane via specific transport systems. The internalized peptides are hydrolyzed by cytoplasmic peptidases.

Proteinases. All milk proteins including whey proteins are available for hydrolysis in starter strains. It could be expected that more accessible proteins in the casein micelle, e.g., κ- and β-casein, are hydrolyzed before α_s-casein.[27] This has been shown in lactococci. *Lb. helveticus* has been shown to attack α_s-casein and partly β-casein, and *Lb. bulgaricus* degrades all the major caseins, β-casein being most susceptible.[28,29] The proteolytic activity of *Str. thermophilus* is lower than that of lactococci and does not affect casein hydrolysis in cheese.[30]

The proteinases of lactococci involved in the first step of casein breakdown are high molecular weight proteins located primarily in the cell wall. Their optimum pH is around 5.5–6.5, their isoelectric point is between 4.4 and 4.55, and they are either activated or stabilized by Ca^{2+} ions.[31] Several studies describe three cell wall–associated proteinases. One of these is thought to be responsible for the bitterness of cheese.

These observations suggest an active role of the lactococcal proteinases in producing bitter peptides of medium size (tri- to hexapeptides) during cheese maturation.[32] The identification and characterization of cell wall–associated proteinases are under investigation. It is likely that the known number of different proteinases may change due to improved knowledge of their specificity and their genetic back-ground.

Because spontaneous irreversibly proteinase-negative variants appear with high frequency, the involvement of plasmid DNA has been studied. Plasmids ranging in size from 13.5 to 100 kilobases (kb) are involved in proteinase production. Studies on proteinase localization have shown that they are attached to the cell wall by an "anchor" present at the C-terminal end of the protein. The removal of this anchor results in the release of the proteinase.[31] In addition, it seems that a membrane-located lipoprotein is involved in the activation of the proteinase. The role of this system, under the control of a gene called *prt M*, is not yet understood.

Peptidases. Casein degradation initiated by a milk-clotting enzyme and proteinases, which produce large peptides, continues with peptidases, which produce smaller peptides and amino acids. A number of peptidases has been described. Aminopeptidase, di- and tripeptidase, an arylpeptidyl-amidase, aminopeptidase P, proline-iminopeptidase, prolinase and prolidase, X-prolyl-dipeptidyl aminopeptidases, endopeptidases, and carboxypeptidases have been found in various lactococci.

Unfortunately, the various studies cannot be compared because the methods used differed. A majority of peptidases are metal enzymes. It has been suggested that citrate and other carboxylic acids affect peptidase activity.[33] This is supported by the fact that in cheese where BD cultures are used, the amino acid nitrogen level is higher than in the control cheese prepared only with *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*. Lactobacilli exhibit a wide range of peptidase activity. Aminopeptidase activity is especially high in *Lb. helveticus*,[34] but dipeptidase and caseinolytic activities do not vary much between *Lb. helveticus*, *Lb. lactis*, and *Lb. bulgaricus* strains.[35] Several peptidases with broad specificities have been isolated from *Lb. casei NCDO* 15 and 2 strains of and *Lb. plantarum*.[36] Being natural contaminants of cheese, these species could have a considerable effect on proteolysis, texture, and taste.[37]. The location of peptidases in the cell has not been determined.

3. Aroma Formation

The flavor compounds produced by lactococci can be divided into two categories: the compounds in products of fermented milk, and the compounds present mostly in matured cheeses. The aroma compounds in fermented milk are mainly organic acids, lactic and acetic acid, produced by *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*. Second, *Lc. lactis* ssp. *lactis* var. *diacetylactis* and *Leconostoc* ssp. produce acetaldehyde, diacetyl, acetoin, and 2, 3-butylene-glycol from citrate. It has been suggested that these arome compounds prevent pyruvate accumulation in the cell. The pyruvate metabolized from citrate by citrate lyase (or citritase) is toxic to the cell when its intracellular concentration is too high. Pyruvate is degraded in the cell in the presence of Mg^{2+}, Na^{2+}, and thiamine pyrophosphate. The lactococcal citrate metabolism pathway consists of enzymes the genetic determinant are in plasmids. Indeed, the studies carried out on this topic have shown that citrate-negative variants of a citrate have always lost a 5.5 megadalton plasmid.[23]

The impact of lactococci on the production of flavor compounds in ripened cheese is much more difficult to determine.[33] One of the reasons for this is that the lactococci play an indirect role in cheese flavor production. The peptidases generate di- or tripeptides and free amino acids, which are further metabolized to volatile compounds. No direct relationship has been established between the amino acid nitrogen and cheese flavor, even though it is known that the former influences the latter. Some key flavor compounds are present at nanogram concentrations, and analytical methods are inadequate. Nevertheless, better knowledge of proteolysis and peptidolysis in cheese, analysis of enzymatic systems of lactococci, and evaluation of different strains used in cheese production might allow us to establish a better correlation between lactococcal activity in cheese and flavor development.

The aroma compounds in Swiss cheese have been reported to be produced by reactions between dicarbonyls and amino acids.[38] The dicarbonyls, glyoxal, methylglyoxal, dihydroxyacetone, and diacetyl have been found in Swiss, mozzarella, and cheddar cheeses and in cultures of *Lb. bulgaricus*, *Lb. casei*, *S. thermophilus*, and *Propionibacterium shermanii*.[39] Many varieties of cheese contain these species.

4. Exopolysaccharide Formation (Ropiness)

Many strains of lactic acid bacteria produce exopolysaccharides (EPS). The form of EPS can be as a capsule closely attached to the bacterial cell or as loosely attached or excreted slime.[40] A comprehensive review has been published by Cerning.[41]

Slime-forming lactic acid bacteria has been increasingly used in the dairy industry. This property has been utilized in Finland since the nineteenth century, especially in the production of a thick viscous fermented milk product, villi. The starters of this product contain mesophilic, slime-forming lactococcal strains together with aroma-producing lactococci and leuconostocs. At the end of the 1980s the production of thermophilic viscous yogurt starter cultures became more common. They are widely used to increase the rheological quality of yogurt and to inhibit syneresis of the coagulum. These starters are used, in some cases, to replace stabilizers in yogurt.

There have also been some attempts to investigate the antitumor activity of slime-forming lactic acid bacteria.[42,43] The role of EPS in this phenomenon has yet to be elucidated.

Production of EPS in mesophilic lactococci has been shown to be plasmid encoded.[44–46] This may explain the instability of slime production, especially in higher temperatures. However viscosity is unstable in thermophilic starter strains, although they do not contain plasmids.[41,47]

EPS-forming bacteria are often considered to be more resistant to bacterio-phages than nonencapsulated ones. This is not the case among mesophilic lactococci, as these viscous strains are hosts for many phages [48] and a certain phage can also dissolve the capsular material of even nonhost strains.[49]

The chemical composition of EPS of mesophilic and thermophilic lactic acid bacteria varies from strain to strain. All of them have been shown to contain galactose and glucose and sometimes hexose-like components and rhamnose (reviewed by Cerning,[41]). In some isolated capsular materials protein has been found, but the amino acid composition of this protein is similar to that of the whey.[50] Toba et al. [51] found that glucose, galactose, rhamnose, glycerol, and phosphorus form a capsular polysaccharide of *Lactococcus lactis* ssp. *cremoris.* It was thought to be a deacylated lipoteichoic acid. Nakajima et al. [52] isolated a phosphorus-containing polysaccharide that contained rhamnose, glucose, and galactose but not glycerol.

5. Production of Inhibitory Components

The observation that lactic acid bacteria have some preserving effect dates back to the turn of the nineteenth century. According to the early research the organic acids from sugar fermentation were responsible for the good keeping quality of fermented foods. Thus, reduction of pH and production of organic acids (lactate, acetate) are the primary inhibitory actions of these bacteria. Few other bacteria are able to grow at pH values achieved by the action of lactic acid bacteria.

Lactic acid bacteria produce other inhibitory substances, although in much smaller amounts. These include hydrogen peroxide, diacetyl, bacteriocins, and secondary reaction products such as hypothiocyanate generated by the action of lactoperoxidase on hydrogen peroxide and thiocyanate. Since many reviews have been written on this topic and there is a chapter in this book about the subject, only general remarks are presented here.

Hydrogen peroxide is generated by different mechanisms by certain lactobacilli,[53] and accumulation of hydrogen peroxide in growth media can occur because lactobacilli do not possess catalase.[54] Antagonistic effect has been demonstrated against *Staphylococcus aureus* [55] and *Pseudomonas* spp.[56]

The second system of inhibition, attributed to lactic acid bacteria and linked to hydrogen peroxide production, is the lactoperoxidase system (LPS).[57] To make this system efficient, certain components have to be present in milk. An enzyme, lactoperoxidase, reacts with two substrates: thiocyanate and hydrogen peroxide. The concentration of lactoperoxidase in milk is 10–30 μg/mL. Thiocyanate (SCN^-), widely distributed in animal secretions, is detected in milk at a concentration varying from 1 to 10 ppm. Hydrogen peroxide (H_2O_2) is produced in milk by lactic acid bacteria, even at low temperatures. Hydrogen peroxide can react with thiocyanate in a reaction catalyzed by lactoperoxidase to form an oxidation product, hypothiocyanate, which inhibits microorganisms.[58]

Diacetyl imparts butter aroma, but it is also well recognized for its anti-microbial action. The inhibitory level by Jay [59] is 200 μg/mL for yeasts and gram-negative bacteria and 300 μg/mL for nonlactic, gram-positive bacteria. A relatively large amount is needed for inhibitory action, and thus the use of it in foods may be problematic.

Bacteriocins are a heterogeneous group of antimicrobial substances in respect to bacteria production bacteria, antibacterial spectrum, mode of action, and chemical properties.[53] Bacteriocins of lactic acid bacteria have been the subject of wide research. The bacteriocins are defined as follows:[60]

They generally have a narrow range of action.
Part of the molecule is a peptide and therefore they are sensitive to proteases.
They are thermostable.

Bacteriocins produced by lactobacilli have been characterized from *Lb. fermentum*,[61] *Lb. helveticus*,[62] *Lb. acidophilus*,[63] and *Lb. plantarum*.[64]

The two bacteriocins produced by lactococci, nisin and diplococcin, are well characterized. Nisin was found in 1928 by Roger and Whittier. The first application in Swiss cheese was done 1951 by Hirsch et al.,[65] and it was found to be effective in prevention of blowing (butyric acid fermentation) caused by clostridia. Nisin is effective against gram-positive species and also against *Clostridium botulinum* spores, which has made it useful in thermally processed foods. Recent investigations have also shown nisin to be inhibitory toward *Listeria mono-cytogenes*, a pathogen of great concern.[66]

Diplococcin, produced by *L. lactis* ssp. *cremoris*, is active only against *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*. As diplococcin is more active against cells in the exponential growth phase than in the stationary phase, it is suggested that its targets include both RNA and DNA.[67]

III. FACTORS INFLUENCING STARTER ACTIVITY

A. Milk as a Growth Medium

Even though lactic acid bacteria are able to grow in milk, milk is not an optimal growth medium. For instance, addition of yeast extract can stimulate the production of lactic acid.[68] Variations in milk lead to modification of the physiological reactions of the microorganisms. It is well known that the origin of the cows, the geographic location, and the stage of location all cause variation in milk components. The average composition of cow's milk is:

Water, 905 g/L
Lactose, 49 g/L
Lipids, 35 g/L
Protein, 34 g/L
Salts, 9 g/L
Other (vitamins, enzymes, etc.) traces

The impact of lactic acid bacteria on the main components of milk involves uptake of fermentable sugars, proteins and peptides, citrate, and their corresponding enzymatic systems. Lactic acid bacteria have a very limited effect on milk fats.

The remaining components of great importance in the nutrition of lactic acid bacteria are, on the one hand, vitamins and, on the other hand, nonprotein nitrogen (NPN). The vitamin requirements vary from species to species. Lactococci require niacin (PP), pantothenic acid (B_5), pyridoxine (B_6), and biotin (H). Thermophilic streptococci require pantothenic acid (B_5) riboflavin (B_2), thiamine (B_1), niacin (PP), biotin, and pyridoxine. Lactobacilli require calcium pantothenate (B_5), niacin (PP) and riboflavin (B_2). In addition, *Lb. lactis*, *Lb. bulgaricus*, and *Lb. acidophilus* require cobalamin (B_{12}).

Nonprotein nitrogen represents 5–70% of the total nitrogen in milk. The constitutive molecules of this fraction have an important role in the nutrition of lactic acid bacteria because of their direct uptake by the cell. The concentration of these components (containing fewer than eight amino acids) is usually too low to provide the required nutrients. Consequently, free amino acids (including free methionine, an essential amino acid) are not present in milk at sufficient levels to allow satisfactory growth of the cells.[68]

B. Inhibitory Compounds in Milk

The antimicrobial effects of milk have long been known, but not until 1927 were the agents identified by Jones and Little as lactenins, and afterwards divided into lactenin 1 (L_1) and lactenin 2 (L_2) by Auclair and Hirsch.[69] These antimicrobial substances were later referred to as red protein, i.e., lactoferrin.[70] Many antimicrobial factors have been identified since then, some derived from cow's blood.

Endogenous or exogenous factors can affect the starter activity in the starter tank or during the dairy process. These include variations in milk composition caused by mastitis or seasonal changes, agglutinins, dissolved oxygen, free fatty acids, inhibitory bacteria, the lactoperoxidase system, lysozyme, lactoferrin, residual sanitizers, and bacteriocins.[6,7,71–74]

Heat processing of milk and good manufacturing practices combined with intensive quality-control procedures have minimized the effect of most of the factors mentioned above. Therefore, they are not reviewed here in detail. The most important sources of problems in the dairy plant are antibiotic residues and bacteriophages. These are discussed further below.

1. Antibiotic Residues

Antibiotics are the most important group of exogenous inhibitory factors because of their common use in the treatment of mastitis in dairy cows. Antibiotics were introduced in the 1940s for mastitis therapy. Today mastitis is still the most serious problem affecting cows, and it causes huge economic losses to dairies. There are also several health aspects associated with the problem, e.g., allergic reactions, intestinal disorders, and development of resistant bacteria.[75,76]

To avoid residues in milk, the manufacturers of veterinary products are generally compelled to specify a withdrawal period for any product. However, individual differences between cows are known to exist, and thus general withdrawal times are not always valid. Strict penalty rules and improved testing systems have reduced the residue levels significantly. The number of antibiotics used is huge and still increasing, varying from country to country.[77] The most widely used group is β-lactam antibiotics and their derivatives, either alone or in combination. Other common antibiotic groups are amino-glycosides, tetracyclines, macrolides, and sulfa drugs.[78,79]

The levels of antibiotics required to inhibit different starter strains are very strain dependent.[12,80] This can be seen in Table 1. Mesophilic cultures are less sensitive to penicillin and spiramycin and more susceptible to streptomycin and cloramphenicol than thermophilic cultures, but streptomycin-sensitive *S. thermophilus* starter strains have been found.[81] Little information is available on the levels of antibiotics required to inhibit leuconostocs or propionic acid bacteria used in hard cheeses. In an experiment to test the actual effect of low antibiotic levels on cheese produced by mesophilic (Edam cheese) and by thermophilic (Emmental cheese) starters, different antibiotics were added to the cheese vat. The results are presented in Table 2.

Table 1 Sensitivity of Thermophilic and Mesophilic Starters to Different Antibiotics

	Starter cultures	
Antibiotic	Thermophilic[a]	Mesophilic[b]
Penicillin, IU/mL	0.004–0.01	0.005–0.01
Tetracycline, μg/mL	0.3–0.5	0.05–0.2
Streptomycin, μg/mL	0.5–5.0	0.5–1.0
Cloramphenicol, μg/mL	0.5–1.0	0.2–0.3
Spiramycin, IU/mL	0.3–0.5	2.0–4.0

[a]Thirty-two *S. thermophilus* strains tested.
[b]Single strains of *Lactococcus lactis* ssp. *lactis*/*cremoris*/*diacetilactis* and three mixed cultures (DL) tested.
Source: A. Mäyrä-Mäkinen, unpublished data.

Table 2 Effect of Different Antibiotics at Low Concentrations on Edam and Emmental Cheese Quality

	Cheese quality	
Antibiotic	Edam[a]	Emmental[b]
Penicillin		
0.003 IU/mL	No defects	No defects
0.005 IU/mL	Tasteless	Off-flavor, abnormal eye formation
0.008 IU/mL	Bitter, uneven body	Strong off-flavor, butyric acid fermentation
0.01 IU/mL	Strong off-flavor, uneven body	Not tested
Spiramycin		
1.01 IU/mL	Tasteless, uneven body, wet surface	Smell of butyric acid, severe off-flavor, uneven eye distribution
5.0 IU/mL	Strong off-flavor, uneven body, slimy surface	Not tested
Streptomycin		
1.0 μg/mL	Abnormal cheese, strong off-flavor	Brown spots in the body, strong off-flavor
Tetracycline		
0.3 μg/mL	Tasteless	Off-flavor
0.7 μg/mL	Tasteless, strong smell on surface	Not tested

[a]Evaluation at 14 weeks.
[b]Evaluation at 3 months.
Source: A. Mäyrä-Mäkinen, unpublished data.

It can be concluded that low levels of antibiotics cause different kinds of defects in cheese: off-flavors, uneven texture, uneven eye formation, and butyric acid fermentation. Effects on propionic acid bacteria could be seen in eye formation of Emmental cheese and in browns-pot defect caused by streptomycin. In fermented milk products the effect of antibiotics is seen in slow or inhibited acid formation and in a decrease in aroma formation.

2. Bacteriophages

Bacteriophages are viruses that specifically infect bacteria. After infecting bacterial cells, they use the cells' enzymes to grow. After some time cells are lysed and bacterial growth is stopped. In the dairy industry, phages of lactic acid bacteria are of considerable economic importance because they represent one of the main causes of fermentation failure. Due to their economic importance, much work has been done to improve our knowledge of phage infection and bacterial phage mechanisms.

Taxonomy. In order to differentiate the phages, various taxonomic criteria have been proposed. The most important of them, proposed for a taxonomic classification, are host range, morphology, serotyping, DNA/DNA hybridization, and structural protein profiles.

1. Host Range: Strain rotation is more easily handled when the host range of a phage is known. But as this criterion cannot be correlated with others, it is a practical parameter rather than a real taxonomic characteristic.

2. Morphology: Phages are submicroscopic particles consisting of a head containing the DNA and a tail. Morphological classification was proposed on the basis of electron microscopic observation. In phages of lactic acid bacteria, various morphologies are observed. The head, with either prolate, small isometric, or large isometric shape, has a size of 40–70 nm. The tail measures between 100 and 500 nm.
3. Serotyping: Antibodies have been prepared against pure phages in order to classify them in various groups. These groups have been compared with host range classes.[82] The results of these studies have been variable, indicating that the serological criteria are not sufficient to classify the majority of the phages.
4. DNA/DNA Hybridization and Structural Protein Profiles: It has been possible to determine five groups of homology for a large number of phages of *Lc. lactis* by DNA/DNA hybridization (Prevots et al., 1990). The details of these five groups are given in Table 3. Groups I and III contain almost 80% of phages and include only virulent phages. Group II contains both virulent and temperate changes. The classification, based on structural protein profiles, allows the grouping of phages corresponding to the five DNA homology groups.[84]

Phage Development and Bacterial Resistance. Phage resistance mechanisms developed by lactic acid bacteria are correlated to the various steps of phage infection. First, the phage recognizes a molecular structure on the cell wall. This receptor allows the phage to adsorb on the cell surface. Second, the phage injects its chromosome inside the bacteria. The DNA penetration is Ca^{2+}-dependent and energy-requiring. In the next step, bacterial DNA is hydrolyzed, and the bacterial metabolism is used by the phage genome to develop new phage particles. In the last step, a lytic enzyme or lysin is synthesized by the phage to make the cells burst. As a rule, an average of 100 phages per bacteria are released into the milk.

Several, lactic acid bacteria have developed mechanisms to resist phage attack.[85] Most of the known mechanisms are coded by plasmids and, therefore, may be transferred from one cell to another. Four mechanisms are well described:

1. Adsorption: Phage-resistant strains have mutated cell-wall structures, recognized as receptors by phages. The phage fails to adsorpt on cell surface and therefore does not infect the strain.

Table 3 Classification of Lactococcal Bacteriophages by DNA/DNA Hybridization

DNA homology group	Percent of phages of the group	Head morphology	Genome size (kb)
I	29	Prolate	19–22
II	21	Small isometric	30–40
III	48	Small isometric	30–35
IV	1	Large isometric	53
V	1	Large isometric	134

Source: Ref.[83].

2. Restriction-Modifications: The restriction-modification system combines restriction enzymes, capable of specific endonucleolytic activity, with a modification enzyme, generally exhibiting specific DNA methylation activity. This methylation protects the DNA from the corresponding restriction enzyme. The unmodified phage chromosome is hydrolyzed by the restriction enzymes as soon as it enters the cytoplasm and, consequently, is degraded.
3. Abortive Infection: In the case of abortive infection, all the phases of the infection occur. But, due to an unknown phenomenon, the burst size is very low. So, each attacked cell releases very few phages in the medium. Little or no disturbance is observed in the growth rate and acidification during cheese making.
4. Lysogenic Immunity: This is observed when the bacterial chromosome harbors the DNA of a lysogenic phage. The prophage probably codes for molecules, which inhibit the development of other related phages and render the strain resistant.

IV. USE OF STARTERS IN THE DAIRY PROCESSES

There have been considerable changes in the cultivation of starters in dairy processing. The starters available are sold in different forms by several starter producers. Starters are the most important factors determining the final quality and properties of the product. Therefore, the selection of starter type and form is very important for the individual dairy plant.

General practical steps in the preparation of starters are discussed here, including the traditional systems (liquid starters), since they are still in practice, besides the increasing use of concentrated starters. The four alternative commercially available forms of starter cultures are shown in Fig. 1.

In the traditional liquid starter system the starter is first cultivated as a liquid stock culture, and the desired volume is reached by successive subcultures. The stock culture comes weekly from a central laboratory or from the plant's own culture. The procedure is thus expensive, laborious vulnerable, and needs skilled personnel to manage it. The starters are easily contaminated during the numerous inoculations or infected by bacteriophages. Still, liquid starters are widely used, especially where local special products are made or where the transportation of starters from a central laboratory is easy and regular.

Freeze-dried cultures can also be used for producing a mother culture or bulk starter in cases where a small amount of starter is needed. They can be stored for several months at -25°C, and thus the plant can use the same production lot for months. In the case of mixed-strain cultures the strains should be freeze-dried separately in order to avoid changes in balance.

The modern systems of concentrated frozen and concentrated freeze-dried cultures have made it possible to directly inoculate the bulk starter or the production process itself. This causes significant savings in labor and material costs in the dairy. The production technology of concentrated frozen starters was developed during the 1960s. These starters require low temperatures during shipment and storage. In 1970 the freeze-drying technique was developed to make use and transportation still easier.

There are several advantages to the use of concentrated and concentrated freeze-dried starters[86,87] they are easy to use, the quality is even, activity is good and can be

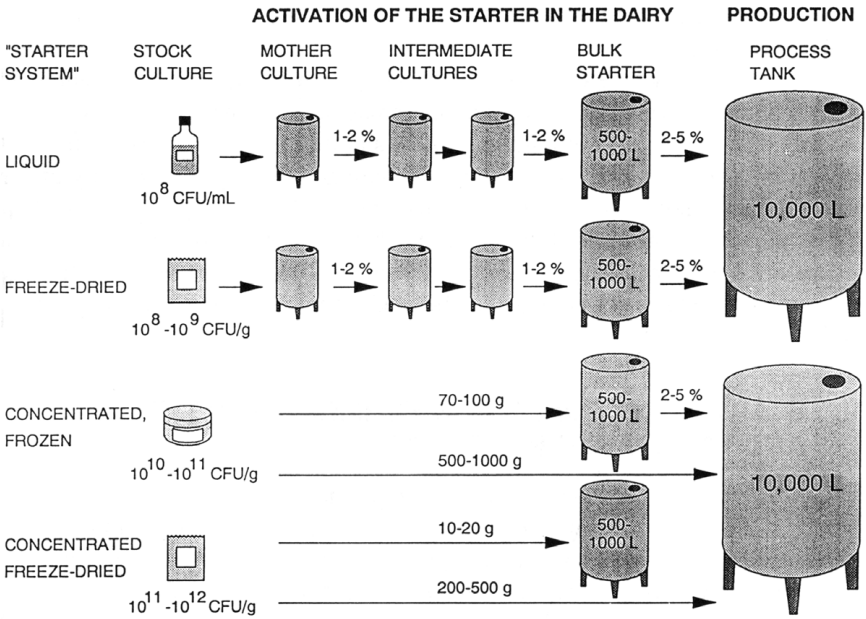

Figure 1 Alternative starter types used in dairy product manufacture and cultivation steps in the dairy process.

tested prior to use, they require less labor and are easily adapted to a five-day production week, and bacteriophage control is easier to manage.

On the other hand, there are also disadvantages.[87] The shipment of frozen starters is precarious since temperature changes affect the starter activity, the storage temperature is critical, and thus the activity has to be controlled in the dairy plant. The selection of starters with respect to quality of the final product is not made by the dairies but by the starter producers. Not all good traditional milk starters are suitable for production as concentrated freeze-dried starters. Despite these disadvantages, starter research is strongly oriented to production techniques and strain selection in order to produce active, directly-to-vat starters.

V. STARTER CULTURES FOR FERMENTED MEAT AND VEGETABLE PRODUCTS

The growth of lactic acid bacteria in milk to produce fermented dairy products is based on a few simple principles: lactic acid bacteria are present, among other bacteria, in several niches. When the physical conditions (such as temperature, water activity, and pH) allow growth, there is a competition between various species. The faster development of lactic acid bacteria and the pH decrease due to acid production lead to microbiologically stable fermented products. These basic phenomena of bacterial ecology have been used to produce meat products such as sausages and fermented vegetables.

For sausage preparation, beside the meat slurry, fermentable sugars, salt, and spices, pediococci such as *P. acidilactici*, *Lb. plantarum*, and *Staphylococcus carnosus* are generally inoculated. The first incubation period (time and temperature adapted to the

technology) allows the bacteria to grow, and the pH decrease is measured. Thereafter the sausages are cooled and aged to obtain the final product.

Widely available fermented vegetables include sauerkraut, pickled cucumbers, and green olives. Usually, cabbage, cucumbers, and olives are fermented with *Lb. plantarum*. *P. cerevisiae*, *P. pentosaceus*, *Leuconostoc mesenteroides*, or *Lb. brevis* can also be used.[88] During the preparation of these products, NaCl brine is added. The combination of NaCl and lactic acid makes the fermented vegetables stable for a long period of time at room temperature. The salinity also controls the growth of lactic acid bacteria, thereby influencing the quality of the end product.

VI. PRODUCTION OF STARTERS ON AN INDUSTRIAL SCALE

The interest in producing concentrated frozen starters began during 1960s with research on mesophilic single strains and especially cheddar cheese starters. Kosikowski[89] and Bergere and Hermier[90] were the first to use neutralization to increase the bacteria count in fermentations about 100-fold in concentrated cultures.

Methods to produce concentrated cultures differ in several ways from traditional ones. Starter strains are grown under strictly controlled conditions in a medium from which the cells are easily harvested into a smaller volume.[87] The process is very stressful for the starter strains and new selection criteria must be used. Only about 25–50% of traditionally used strains are suitable for the production of concentrated, freeze-dried cultures.[91]

Starter concentrate production can be divided into the following general steps, which have been described in detail by Porubcan and Sellars,[92] Gilliland,[87] and Tamine and Robinson:[80]

Preparation of the inoculum
Preparation of the media
Fermentation at constant pH
Harvesting the culture
Adding the cryoprotectant
Freezing
Freeze-drying
Packing and storing

A. Fermentation of Starter Cultures

To produce active and storage-stable concentrated cultures, several strain-dependent factors have to be checked concerning the growth medium and growth conditions. Industrial-scale production of cultures involves batch fermentations, which are simpler and more convenient than continuous fermentation processes. There are several problems with using continuous fermentations: undesirable contamination is possible, complex equipment is associated with difficulties in production schedules, and bacteriophage problems have also been reported.[93]

1. Growth Medium

To choose the growth medium, the following aspects must be considered: cost, ability to produce a high number of cells (about 10–15 times higher cell densities that in liquid milk culture) with high activity, and effect on the harvesting methods.[86] It is generally

accepted that some milk solids should be included in the growth medium to ensure the synthesis of necessary enzymes for starters to perform well in milk and to maintain a balance between strains in multiples-strain starters when fermented as a mixed culture.[94]

The use of cheese whey and whey permeate as growth media has been investigated primarily because they are inexpensive and contain nutrients that are used by starter strains for growth. Whey alone is not rich enough for maximum growth, and much of the research has involved supplementing whey with extra nutrients. In addition, improper heating causes precipitation in the medium, and clarification might be necessary to avoid this material in concentrated starter culture.[93,95]

Skim milk has been the most common medium for lactococci, and the difficulties in harvesting have been solved by adding sodium citrate to solubilize milk proteins.[94]

A variety of different nutrients are needed for starter strains in the culture medium. Porubcan and Sellars.[92] divided them into four groups, and the first group of complex nutrients—skim milk, whey, yeast extract, and peptones—are used to satisfy the complex demands of starters if there is no way to determine the exact growth factors during the fermentation process.

With certain nutrients or additives the resistance of cultures against subsequent concentration/freezing/drying processes can be improved. The methods are strain-dependent. The activity and the bacterial count of *Lb. bulgaricus* after freezing at $-196^{\circ}C$ increases considerably if the cells have been grown in an appropriate medium supplemented with Tween 80.[96] The same results have been reported for cultures frozen at $-17^{\circ}C$. Oleic acid in Tween 80 was identified as the effective component to increase process stability by raising the levels of a C_{19} cyclopropane fatty acid in the lipid fraction of cells.[97] Most fatty acids of bacterial cells are located in the cell membrane, and it can be assumed that the membrane composition is very important for the cells to survive freezing, perhaps by increasing membrane flexibility.[87] It has also been noted that lactococci survive freezing at $-196^{\circ}C$ better than lactobacilli, regardless of the growth medium. This could be because lactococci naturally contain higher levels of C_{19} cyclcopropane fatty acid.[98] The ratio of unsaturated to saturated fatty acids in cell membranes also seems in some cases to be related to the ability of lactococci and lactobacilli to survive freezing. By increasing the ratio, survival at $-17^{\circ}C$ has improved.[97]

The addition of 100 μM—1 mM calcium has been shown to influence the freezing resistance of lactobacilli and change the cell morphology from long chains to short individual cells. Manganese and magnesium did not have this effect.[99,100]

2. Growth Conditions

The conditions during the fermentation affecting the growth and activity of cultures are temperature, pH, mixing (oxygen content), and type of neutralizer used. Also, the optimum cooling/harvesting time relative to the growth curve has to be considered.

Usually the optimum growth temperature of the species is used in fermentation,[101,102] but few research results have been published on the effect of temperature on process stability. When growing mixed cultures, Bauman and Reinbold[103] reported better freezing stability at $-20^{\circ}C$ when a temperature of $32^{\circ}C$ had been used during fermentation. With thermophilic cultures it has been noticed that, compared to the optimum growth temperature, a decrease or increase in the growth temperature strain dependence affects the dechaining of cultures and thus also process stability in freezing and freeze-drying (A. Mäyrä-Mäkinen, unpublished data).

Maintaining the pH of the growth medium at the optimum level increases the number of cells.[104,105] Both the optimum pH of the culture and type of neutralizer used are of importance. Most research has been done with lactococci, whose optimum pH is in the range of 6–6.5.[93,106,107] Ammonium hydroxide seems in general to be the best neutralizer in order to achieve higher cell yields in mixed cultures[93] and more freezing-resistant cultures at −30°C.[108] The optimum pH of thermophilic lactobacilli, *Lb. helveticus*, *Lb. lactis*, and *Lb. bulgaricus*, is 5.4–5.8, depending on the strain. Higher yields of these species are obtained with ammonium hydroxide as the neutralizer (A.Mäyra-Mäkinen, unpublished data).

Cooling and harvesting at certain stages of the growth curve are critical for some cultures. For lactococci, harvesting is recommended at the end of the logarithmic growth phase. *Str. thermophilus* cultures lose activity fast if cooling and harvesting is delayed to the stationary phase. On the other hand, many lactobacilli can be harvested irrespective of the growth phase without losing activity (A.Mäyrä-Mäkinen, unpublished data). Increasing the growth seems to be a combination of several factors, of which the formation of lactate salts is considered to be the most important.[92] To reach the maximum bacterial level in the fermentation, the factors limiting growth have to be considered, in addition to the type and amount of nutrients adequate for optimal growth.

In order to maintain constant pH continuous agitation is needed. As a result, oxygen toxicity has been observed in culturing some lactococci.[109] Oxygen can also cause the production of hydrogen peroxide by some starter strains, which can be autoinhibitory. By adding catalase or other reducing agents, on the accumulation of H_2O_2 can be prevented.[110] Other toxic metabolites can be formed in the growth medium; D-leucine formation has been reported in lactococcal cultures by Gilliland and Speck.[111] Sparging carbon dioxide can be an effective way to avoid the toxicity of oxygen and is actually needed for optimal growth of some starters.[101,106]

It can be concluded that the basis for active, process-stable culture is built during the fermentation by modifying the growth media and conditions so that they are strain-dependent. Especially important factors to be checked for industrial production are:

Dechaining effect of certain components in medium
Temperature optimum to produce process-resistant strains (not always the growth optimum)
pH optimum for growth and further processing
Harvesting time at certain point of growth curve
Process resistance as selection criterion for starter strains

B. Concentration of Fermented Cultures

Centrifugal separation or membrane processes can be used for harvesting cells from the medium. Centrifugation is mostly used on an industrial scale because the low viscosity of the medium, the properties of the cells, the large cell size, and higher temperatures favor this technique. The temperature is usually kept between 5 and 15°C, depending on the strain.[92]

Very little has been published about concentration of thermophilic lactobacilli. According to Porubcan and Sellars,[92] it is difficult to concentrate *Lb. bulgaricus*, *Lb. lactis*, and *Lb. acidophilus* from milk-based cultures by centrifugation, even with the addition of citrate. Porubcan and Sellars[92] also reported industrial-scale ultrafiltration

of lactococci. An approximate 12-fold concentration can be reached by ultrafiltration without any cell damage caused by heat developing during the process. Microfiltration processes with ceramic filters are becoming an important method, especially in concentrating process-sensitive cultures.

C. Handling of the Concentrate

Cryoprotectants have long been used to improve the ability of culture concentrates to survive freezing, frozen storage, and freeze-drying. Most of the research on freeze-dried cultures has been done with lactococci. The resistance of cultures against the deleterious effects of freeze-drying can be improved by cryoprotectants.[112] A variety of different cryoprotectants are used[113] but the most common ones in industrial production are lactose or sucrose (7%), monosodium glutamate (5%), and ascorbate in a milk or water base. Glycerol is widely used in frozen cultures, but there seems to be a variation among cultures, and for some cultures glycerol is not effective.[114] Lactose (7.5%) has been used with good results, but for some cultures again no effect has been seen.[107] According to several reports it seems that cryoprotectants are not needed if the concentrate is active, freezing is fast ($>1^{\circ}C/s$), and storage is at $-196^{\circ}C$.[105,115] Freezing can affect the activity of cultures strain-dependently. The most efficient and widely used method is fast-freezing in liquid nitrogen in the form of pellets, which are easy to use as a frozen concentrate or to freeze-dry.[22,116] The use of liquid nitrogen is expensive. Therefore, in many cases $-40^{\circ}C$ freezing and storing temperature is also used.

The extensive report of Morichi[112] concerning the mechanisms and cryoprotectants involved in freeze-drying gives a view of this complex area. Although possible mechanisms of cryoprotection have been proposed by Fennema et al.,[113] further research is needed to improve the "freeze-drying resistance" of cultures.

The pH of the concentrate affects the activity during storage. The activity of a mesophilic mixed-culture concentrate with a pH of 5.2 was lower than that of a concentrate with a pH of 6.6 after frozen storage.[117] The optimum concentrate pH for lactobacilli is 5.4–5.8. A lower pH does not seem to have any effect on the activity after freeze-drying. *Str. thermophilus* cultures, on the other hand, lose activity easily if the pH of the concentrate is below the optimum 6.2–6.6 (A.Mäyrä-Mäkinen, unpublished data).

The activity of a culture can be maintained if storage is carried out under recommended conditions. The shelf life depends on the form of starter culture (frozen or freeze-dried) and the storage temperature: the lower the temperature, the longer the shelf life. Frozen concentrates are stored at $-40^{\circ}C$, and activity remains good for at least 6 months. Freeze-dried products are stored at -20 to -40°, and short-term refrigeration does not usually decrease activity. Again, there are great differences in storage stability between cultures.

VII. FUTURE TRENDS

As explained in this chapter, as far as technology is concerned, the important characteristics of the bacterial strains are being increasingly well understood and measured. The studies carried out in this area have been done mostly with pure strains. Knowledge needs to be improved in the understanding of the global behavior of a culture composed of several strains. Studies must focus on the relationship between the constitutive strains of

a mixture. Understanding the symbiotic and inhibitory phenomena between strains is of prime importance for the control of the cultures used in fermented products.

The second main direction followed by researchers is in genetics. These programs aim at implementing new characteristics in technologically interesting strains. For example, besides acidification, it would be useful to have strains producing bacteriocins in order to manufacture fermented products in which raw materials should not be heat treated, such as sausage, silage, or vegetable products, and where the contaminant level could be reduced by the action of modified lactic acid bacteria. To make these techniques feasible, one must achieve the following:

Identify the corresponding genetic determinant of certain physiological characteristics
Use methods that allow a stable transfer of genes
Dispose of vectors donor and recipient strains of the same species in order to obtain a GRAS microorganism
Solve regulatory problems and constraints related to genetically modified microorganisms

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6

The Genus *Enterococcus*: Biotechnological and Safety Issues

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I. INTRODUCTION

The enterococci are lactic acid bacteria (LAB) that are important in environmental, food, and clinical microbiology. They are ubiquitous microorganisms, but have a predominant habitat in the gastrointestinal tracts of humans and animals.[84] They occur as natural contaminants on meats as a result of contamination from the gastrointestinal tract at the time of slaughter. Enterococci are also of technological importance in the production of various European fermented foods such as sausages and cheeses, either where they are purposefully added to the product as starter cultures[165] or where their presence results from environmental contamination. As a result of their natural association with the gastrointestinal tract as well as functional and technologically desirable properties, some strains are also used successfully as probiotics.[6,117]

The detrimental activities of enterococci are related to spoilage of foods, especially meats and, more importantly, the fact that certain *Enterococcus* strains can cause human disease. Enterococci are typical opportunistic pathogens that may cause infections, especially in the nosocomial setting in patients who have underlying disease. Over the last two decades, enterococci have emerged as important nosocomial pathogens, and this rise in their association with human disease can be explained in part by their increasing resistance to antibiotics as well as their promiscuity regarding transfer of genetic material.[6,84,302] This ambiguous nature of enterococci makes them, on the one hand, desirable for use as starter cultures in food production or as probiotics, while, on the other hand, they give rise to concern because of the potential transfer of antibiotic resistance, the possible presence of virulence factors, and their role in human disease.

II. TAXONOMY

The genus *Enterococcus* was proposed by Thiercelin and Jouhaud[1] for gram-positive diplococci of intestinal origin. Andrewes and Horder[2] classified potentially pathogenic bacteria from a patient with endocarditis as *Streptococcus faecalis*. Because of their close resemblance to strains isolated from the human intestine, the species epithet *faecalis* was suggested. Lancefield[3] developed a serological typing system for streptococci in which those of fecal origin possessed the group D antigen. This correlated with the grouping of Sherman,[4] who proposed a new classification scheme for the genus *Streptococcus* that separated it into four divisions designated pyogenic, viridans, lactic, and enterococcus. The enterococcus group included *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus bovis*, and *Streptococcus equinus* as the enterococcal or group D strains. This explains why the history of enterococci cannot be separated from that of the genus *Streptococcus*.[5,6]

Members of the genus *Streptococcus* formerly grouped as faecal streptococci or Lancefield's group D streptococci were subdivided into three separate genera: *Streptococcus*, *Lactococcus*, and *Enterococcus*. This was first based on DNA : DNA and DNA : rRNA hybridization studies, modern classification techniques, and serological studies,[7–9] and the separation was confirmed on the basis of 16S rRNA sequence analysis.[10] The typical pathogenic species remained in the genus *Streptococcus* and, with the exception of *S. thermophilus* and, more recently, *Streptococus macedonicus*,[11,12] were separated from the nonpathogenic and technically important species of the new genus *Lactococcus*.[5] The fecal streptococci that were associated with the gastrointestinal tract of humans and animals with some fermented foods and with a range of other habitats constitute the new genus *Enterococcus*.

Enterococci belong to the Firmicutes with low mol% G + C content in the DNA, the so-called clostridial subdivision of the gram-positive bacteria, together with the other genera of LAB: *Aerococcus*, *Carnobacterium*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Melisococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*.[5,13,14] Phylogenetically, the closest relatives of the enterococci are the genus *Vagococcus* followed by *Carnobacterium*, *Tetragenococcus*, *Aerococcus*, *Alloiococcus*, *Dolosigranulum*, *Facklamia*, *Globicatella*, and *Abiotrophia*,[15] while the streptococci, lactococci, and lactobacilli are more distantly related.[14]

Since 1984, chemotaxonomic and phylogenetic studies have resulted in the assignment of more than 20 species to the genus *Enterococcus* (for reviews, see Refs.[5,13,14,16]), but the actual number fluctuates from time to time as individual species are reclassified or new taxa are discovered. For example, *E. pallens*, *E. gilvus*, *E. canis*, and *E. phoeniculicola* were only described recently.[17–19] The species *Enterococcus flavescens* [20] appears to be identical to *E. casseliflavus*, which has nomenclatural priority, and Descheemaeker et al.[21] could not distinguish between the two using either protein analysis or PCR-based typing.[14] *Enterococcus solitarius* [22] was shown to be more closely related to the genus *Tetragenococcus*.[23,24] De Graef et al.[18] showed that *E. porcinus* is a junior synonym of *E. villorum*. Moreover, the 16S rDNA sequences of a possible new species, *E. azikeevi*, has been submitted to GenBank (GenBank accession no. AJ309563), but this species has not been further described. The phylogenetic relationship of the different species within the genus *Enterococcus* has been determined by comparative sequence analysis of their 16S rRNA genes. A 16S rRNA-based

phylogenetic tree of *Enterococcus* species is depicted in Fig. 1. Based on these data, the following species groups can be distinguished:

1. ***E. faecium*** group: *E. faecium*, *E. durans*, *E. hirae*, *E. mundtii*, *E. villorum*, *E. canis*, *E. azikeevi*
2. ***E. avium*** group: *E. avium*, *E. malodoratus*, *E. pseudoavium*, *E. raffinosus*, *E. gilvus*
3. ***E. gallinarum*** group: *E. gallinarum*, *E. casseliflavus*, *E. flavescens*
4. ***E. dispar*** group: *E. asini*, *E. dispar*, *E. pallens*
5. ***E. saccharolyticus*** group: *E. saccharolyticus*, *E. sulfureus*

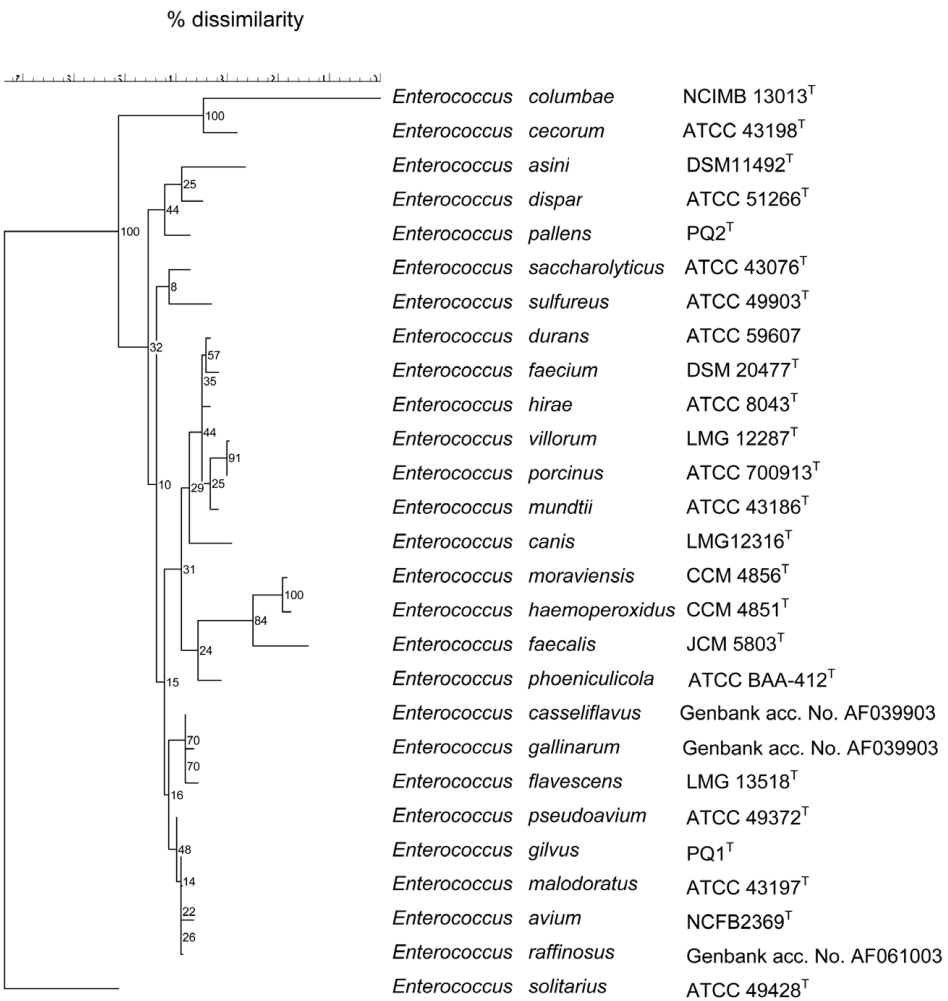

Figure 1 Distance matrix tree showing the phylogenetic relationships of *Enterococcus* species based on 16S rRNA sequence comparisons. *E. solitarius* was used as the outgroup, and the bootstrap probability values (%) are indicated at the branch points (200 tree replications).

6. ***E. cecorum*** group: *E. cecorum*, *E. columbae*
7. ***E. faecalis*** group: *E. faecalis*, *E. haemoperoxidus*, *E. moraviensis*, *E. ratti*

III. IDENTIFICATION

The genus *Enterococcus* is found together with the genera *Melissococcus*, *Tetragenococcus*, and *Vagococcus* within the family Enterococcaceae.[25] Members of the genus *Enterococcus*, like those of the genera *Streptococcus* and *Lactococcus*, are catalase-negative, gram-positive cocci which are arranged in pairs or short chains. Within the chains, the cells are frequently arranged in pairs and are elongated in the direction of the chain. Endospores are absent. *E. gallinarum* and *E. casseliflavus* are motile; all others are nonmotile. *E. casseliflavus*, *E. mundtii*, *E. sulfureus*, *E. pallens*, and *E. gilvus* are yellow-pigmented. All enterococci are facultatively aerobic chemo-organotrophs with a fermentative metabolism. They have a homofermentative lactic acid fermentation, with L(+)-lactic acid as the predominant end product of glucose fermentation. The traditional or "old" *Enterococcus* species (*E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. mundtii*) can be easily distinguished from other gram-positive, catalase-negative, homofermentative cocci such as streptococci and lactococci by their ability to grow both at 10°C and 45°C, in 6.5% NaCl, in the presence of 40% bile, and at pH 9.6 (Table 1). However, many of the more recently described or "new" *Enterococcus* species vary in their physiological properties from those of the typical enterococci (Table 1). Thus, with the increasing number of newly described species, the traditional phenotypic identification using genus-specific characteristics has become exceedingly difficult, if not impossible.[18,26] The Voges-Proskauer (VP) reaction and acid production from ribose have been suggested to have high differential value, especially in discriminating enterococci from streptococci.[5,14] However, De Graef et al.[18] showed that these tests are not universally applicable, especially because in their study on *E. canis* the strains from this species were mostly VP-negative.

Reliable identification of the genus *Enterococcus* and its species thus ultimately relies on the use of a combination of phenotypic, genotypic, and phylogenetic information in a polyphasic taxonomy approach as described by Vandamme et al.[27] A variety of genotypic methods have been used successfully to identify enterococci to genus or species level, and these are reviewed by Domig et al.[28] For differentiation between enterococci and lactococci on the genus level, Deasy et al.[29] described a rapid PCR-based method based on amplification of a region of the 16S rDNA gene. They showed that using this method they could accurately separate enterococci from streptococci, lactococci, pediococci, and lactobacilli. Ozawa et al.[30] were able to accurately identify *Enterococcus* species by PCR amplification and sequencing of a conserved internal fragment of the D-alanine : D-alanine ligase genes (*ddl*). Tyrrell et al.[31] used restriction fragment length polymorphism of the 16S/23S intergenic spacer region to distinguish the *Enterococcus* species, although with some species, e.g., *E. avium* and *E. pseudoavium*, such a differentiation was not possible. Baele et al.[32] used tRNA intergenic spacer PCR for the identification of enterococci species. Williams et al.,[24] Descheemaeker et al.,[21] Quednau et al.,[33] Andrighetto et al.,[34] Gelsomino et al.,[35] and Vancanneyt et al.[36] showed that *Enterococcus* species can be differentiated quite well by RAPD-PCR, while sequencing of the 16S rRNA gene yields accurate species identification and can aid in the description of new *Enterococcus* species (see above).

Table 1 Characteristic Physiological Properties of Validly Described *Enterococcus* Species

	Growth at		Growth in the presence of					
Species	10°C	45°C	pH 9.6	6.5% NaCl	40% Bile	0.04% Sodium azide	Esculin hydrolysis	Group D antigen
E. asini	(+)	(+)	n.d.	−	+	n.d.	+	+
E. avium	V	+	+	V	V/+	n.d.	+	+
E. casseliflavus	+	+	+	V/+	+	+	+	+
E. cecorum	−	+	(+)	−	(+)	−	+	−
E. columbae	−	n.d.	n.d.	−	(+)	−	+	−
E. dispar	+	−	n.d.	+/−	+	−	+	−
E. durans	+	+	+	+	+	+	+	(+)
E. faecalis	+	+	+	+	+	+	+	+
E. faecium	+	+	+	+	+	+	+	V
E. flavescens	V/−	V/+	n.d.	+	+	+	+	+
E. gallinarum	+	+	+	+	+	+	+	+
E. gilvus	+	+	n.d.	+	+	n.d.	+	+
E. haemoperoxidus	+	−	n.d.	+	+	+	+	+
E. hirae	+	+	+	+	+	+	+	V
E, malodoratus	+	−	+	+	+	n.d.	+	+
E. moraviensis	+	−	n.d.	+	+	+	+	+
E. mundtii	+	+	+	+	+	+	+	+
E. pallens	+	+	n.d.	+	+	n.d.	+	+
E. phoeniculicola	n.d	n.d	n.d.	−	−	n.d.	n.d.	n.d.
E. porcinus	+	+	n.d.	+	n.d.	n.d.	+	+
E. pseudoavium	+	+	+	+/−	V/+	n.d.	+	−
E. raffinosus	(+)	+	+	+	V/+	n.d.	+	n.d.
E. ratti	+	+	n.d.	+	n.d.	n.d.	+	(+)
E. saccharolyticus	+	+	n.d.	(+)	+	n.d.	+	−
E. solitarius	+	+	n.d.	+	+	n.d.	+	+
E. sulfureus	+	−	n.d.	+	+	n.d.	+	−
E. villorum	n.d.	n.d.	n.d.	+	+	+	+	n.d.

n.d., not determined, (+), weak positive; V, variable; +/−, differing reports in literature.

IV. HABITAT

A. Environment

Enterococci occur in a wide variety of environmental niches, including soil, surface waters, waste waters, and municipal water treatment plants, on plants, in the gastrointestinal tract of warm-blooded animals (including humans), and, as a result of association with plants and animals, in human foods.[6] Certain *Enterococcus* species are known to be typically associated with plants, i.e., the yellow-pigmented *E. mundtii* and *E. casseliflavus*.[37] On plants, enterococci occur in a truly epiphytic relationship.[38] The early studies on the characteristics of the epiphytic life of enterococci (fecal streptococci) on plants by Mundt et al.[38] were performed before the genus *Enterococcus* was redefined by Schleifer and Kilpper-Bälz;[7] however, modern taxonomic studies based on molecular biological techniques for classification and species identification by Ott et al.[39] and Müller et al.[40] validated this epiphytic

relationship, and enterococci occurring on plants were identified as *E. faecium*, *E. faecalis*, *E. casseliflavus*, *E. mundtii*, and *E. sulfureus*. The majority of the isolates in the study of Müller et al.[40] however, possessed a 16SrDNA genotype uncommon to *Enterococcus* species described at the time of the study.

B. Gastrointestinal Tract

Enterococci are well known to occur as part of the natural microflora of the intestinal tract of warm-blooded animals and humans and constitute a large proportion of the autochthonous bacteria associated with this ecosystem. *E. faecalis* is often the predominating *Enterococcus* sp. in the human bowel, although in some individuals and in some countries, *E. faecium* outnumbers *E. faecalis*.[5,41] Numbers of *E. faecalis* in human faeces range from 10^5 to 10^7/g compared with 10^4 to 10^5/g for *E. faecium*.[42,43]

Although *E. faecalis*, *E. faecium*, and *E. durans* are frequently isolated from human feces, they are less prevalent in livestock such as pigs, cattle, and sheep.[44] In a study by Devriese et al.[45] *E. faecalis* was isolated from feces of pre ruminant calves and ruminating young cattle and dairy cows, and *E. faecium* from pre ruminant calves, but not from ruminating young cattle or dairy cows. *S. bovis* was the predominant group D organism isolated from feces of dairy cows. *E. faecalis*, *E. faecium*, *E. hirae*, and *E. cecorum* were the enterococci most frequently isolated from pig intestines, while *E. faecium* predominated in fecal samples.[44,46] The intestinal microflora of young poultry contained principally *E. faecalis* and *E. faecium*, but *E. cecorum* predominated in the intestine of chickens over 12 weeks old.[47] *E. columbae* is an important member of the gut flora of pigeons, while *E. hirae* frequently occurs in the intestine of pigs but may also occur in the gut of poultry, cattle, cats, and dogs.[48] *E. durans* has been isolated from humans, chickens, and calves, and *E. malodoratus* is often found in the tonsils of cats. The habitat of the members of the *E. avium* species group (*E. avium*, *E. malodoratus*, *E. raffinosus*, and *E. pseudoavium*) otherwise is largely unknown.[14,45]

C. Foods

1. Meats

The presence of enterococci in the gastrointestinal tract of animals clearly leads to a high potential for contamination of meats at the time of slaughter. In raw meat products, *E. faecalis* was shown to be the predominant isolate from beef and pork cuts in one study,[49] while in another both *E. faecium* and *E. faecalis* were the most predominant *Enterococcus* spp. isolated from pig carcasses.[50] These pig carcasses from three different slaughter plants contained mean counts of 10^4–10^8 enterococci per 100 cm^2 of carcass surface throughout processing.[50] Devriese et al.[51] showed that *E. faecium*, *E. faecalis*, and to a lesser extent *E. hirae* and *E. durans* occurred in meat and prepared meat products. In a study on poultry, *E. faecalis* predominated among the gram-positive cocci isolated from chicken samples collected at abattoirs.[52] Capita et al.[53] found enterococci to occur at a mean count of log 2.72 CFU/g of chicken carcasses from five retail outlets in Spain. Enterococci were also consistently isolated from beef, poultry, or pig carcasses or fresh meat cuts in studies of antibiotic resistance of enterococci.[54–58]

Enterococci not only may contaminate raw meats, but can be associated with processed meats. Cooking of processed meats may confer a selective advantage on enterococci as these bacteria are known to be among the most thermotolerant of the

non-sporulating bacteria.[59,60] After surviving the heat-processing step, both *E. faecalis* and *E. faecium* have been implicated in spoilage of cured meat products such as canned hams and chub-packed luncheon meats.[61–64] Enterococci are also isolated from certain types of fermented sausages, particularly the dry fermented sausages known as chorizo[65–67] and espetec[68] produced in Spain, in Italian sausages,[69] or in sausages such as salami and Landjäger produced in many European countries. Salami and Landjäger were shown in one study[70] to contain enterococci at numbers ranging from 10^2 to 10^5 CFU/g. Thanks to their competitive advantage over other microbiota in meat fermentations, the enterococci may survive and contribute to the fermentation of meat products. In addition, some enterococcal strains have the ability to produce enterocins harboring antimicrobial activity against pathogens and spoilage microorganisms of meat concern. Such enterocin-producing enterococci or their purified metabolites may be applied as extra hurdles for preservation in sausage fermentation and in sliced-vacuum packed cooked meat products, thereby preventing the outgrowth of *Listeria monocytogenes* and slime-producing lactic acid bacteria.[71]

2. Cheese

Enterococci occur in many traditional European cheeses manufactured in mostly Mediterranean countries from raw or pasteurized milk.[72–83] The source of enterococci in milk and in such cheeses is thought to be the feces of dairy cows, contaminated water, or milking equipment and bulk storage tanks[84] as well as natural milk starters.[85] The isolation of enterococci from natural milk starters can be explained by their heat resistance; natural milk starters are made by pasteurising milk at 42–44°C for 12–15 hours, thus promoting the thermotolerant bacteria present, which include *S. thermophilus* strains and *Enterococcus* spp.[85] Strains belonging to the species *E. faecalis*, *E. faecium*, and *E. durans* are most often isolated from such cheeses (Table 2), and these may contribute to ripening and product flavor.[34,76,77,80–82,86–88]

Numbers of enterococci in cheese curds range from 10^4 to 10^6 CFU/g, and in the fully ripened cheeses from 10^5 to 10^7 CFU/g (Table 2). Enterococci can grow in the restrictive environment of high salt content and low pH of the cheese[72,75,87,89] and contribute to the ripening and aroma development of these products due to their proteolytic and esterolytic activities, as well as the production of diacetyl.[72,81,90–95]

3. Fermented Vegetables

Enterococci occur in a variety of fermented vegetables, but it is often not clear whether they originate from the plant material itself or as environmental contaminants. Enterococci have been isolated also from Spanish-style green olive fermentations,[96–101] in which *E. faecalis* is a frequent contaminant, and they frequently occur in retail fermented olives. De Castro et al.[97] suggested that lactic acid bacteria growing at the beginning stages of the olive fermentation are important for improving the hygiene of the product. However, not all of the lactic acid bacteria are suited to grow at the relative high pH conditions resulting from alkaline treatment of the olive grapes to hydrolyze the bitter glucoside oleuropein. Because of their tolerance to the high pH values and salt concentration used in the olive brine, the enterococci appear to be well suited for growth at these conditions.[97] It has also been suggested that enterococci can use the antimicrobial compound oleuropein in olive grapes as a growth substrate,[102] thus lowering the toxicity of the fermentation medium for growth of other LAB. In addition, the enterococci, especially *E. faecalis* strains, have also been associated with African fermented sorghum

Table 2 Numbers and Predominance of *Enterococcus* spp. in Cheeses from Mediterranean Countries

Cheese	Country of origin	Milk source	Enterococci in curd (log CFU/g)	Enterococci at end of ripening (CFU/g)	Predominant bacteria in end product (% of isolates)	Ref.
White-brined cheese	Greece	Raw goat's milk or mixed goat's and ewe's milk	4.0	6.7	*L. plantarum* (47%)[b] *E. faecium* (12%) *L. paracasei* subsp. *paracasei* (10%) *E. faecalis* (9%)	86
Kefalotyri cheese	Greece	ewe's milk, cow's milk or mixed ewe's and goat's milk	4.9	5.8	*E. faecium* (35.6%) *L. plantarum* (18.4%) *L. casei* subsp. *casei* (15.8%) *E. durans* (9.2%) Pediococci (9.2%)	75
Teleme cheese	Greece	Pasteurized ewe's milk	n.r.[a]	n.r.	Lactobacilli Leuconostocs Enterococci	86
Orinotyri cheese	Greece	Raw ewe's milk	n.r.	6.8	Lactococci, enterococci, leuconostocs	83
La Serena ewe's milk cheese	Spain	Raw ewe's milk	6.2	7.2	Lactobacilli Leuconostocs Enterococci	74
Manchego cheese	Spain	Raw ewe's milk	n.r.	n.r.	Enterococci	72

Cebreiro cheese	Spain	Raw cow's milk	n.r.	6.5	*E. faecalis* (30.1%) *E. faecalis* (var. *liquifaciens*) (11.9%) *Lact. lactis* (19.0%) *W. (Leuc.) paramesenteroides* (7.9%) *Leuc. mesenteroides* subsp. *mesenteroides* (6.3%) *E. faecium* (4.8%)	81
San Símon cheese	Spain	Raw cow's milk	5–6	6–7	*E. faecalis*, *E. faecium*, *E. durans*, *Staph.* spp. *Micrococcus* spp.	301
Tetilla cheese	Spain	Raw cow's milk	n.r.	7.3	*E. faecalis*, *L. casei* subsp. *casei*, Leuconostoc *mesenteroides* subsp. *mesenteroides*	82
Caprino d' Aspromonte	Italy	Raw or heated goat's milk	4–6	5–7	Enterococci, lactobacilli, mesophilic and thermophilic cocci	80
Serra cheese	Portugal	Raw ewe's milk	n.r.	n.r.	*Leuc. lactis*, *Lact. lactis*, *Leuc. mesenteroides* subsp. *mesenteroides*/*dextranicum*, *E. faecium*	76
Picante da Beira Baixa cheese	Portugal	Mixture of raw goat's and ewe's milk	n.r.	n.r.	*E. faecium*, *E. faecalis*, *E. durans*, *L. plantarum*, *L. paracasei*	87

[a]n.r. = not reported.
[b]*L.* = *Lactobacillus*; *E.* = *Enterococcus*; *Lact.* = *Lactococcus*; *Leuc.* = *Leuconostc*; *W.* = *Weissella*, *Staph.* = *Staphylococcus*.

foods.[103,104] In our own studies on traditional fermented African foods, enterococci were also associated with the fermentation of products such as Hussuwa made from sorghum in the Sudan and Okpehe made from locust beans in Nigeria. In these foods, the enterococci contribute only a minor part of the microbial population associated with the fermentation (approximately 10% of the isolates from Hussuwa), and most isolates consist of *E. faecium* strains.[105]

V. BIOTECHNOLOGICAL IMPORTANCE OF ENTEROCOCCI

A. Bacteriocin Production

Many enterococci isolated from the environment or from foods were shown to be bacteriocinogenic. The bacteriocins from enterococci are usually referred to as enterocins, and generally these exhibit activity towards listeriae.[106] The food origin of many enterocin producers and the activity towards the important foodborne pathogen *L. monocytogenes* has prompted a great amount of research for using bacteriocin-producing enterococci as "protective cultures" or their purified bacteriocins in the biopreservation of foods (see below).

1. Enterocin Classification and Characteristics

Bacteriocins are microbially produced, membrane-active peptides with antimicrobial activity, usually against closely related strains[107] (see also Chapter XX). Their genetics, production, mode of action, immunity, and secretion mechanisms have been reviewed previously.[107–112] The currently most widely used classification system of bacteriocins produced by LAB is that of Nes et al.[111] who grouped these bacteriocins into three classes. Class I bacteriocins are ribosomally synthesized lantibiotics that undergo extensive post-translational modification to produce an active peptide. Lantibiotics contain the unusual amino acids lanthionine and β-methyllanthionine.[107,111] Class II bacteriocins are small (4–6 kDa), heat-stable bacteriocins which were divided into three subgroups by Nes et al.[111]: class IIa comprises the pediocin-like bacteriocins that contain a conserved YGNGVXC amino acid motif at the N-terminus and which generally have a strong anti-*Listeria* effect, class IIb includes the two component bacteriocins, and class IIc consists of bacteriocins that are secreted via the *sec*-pathway or preprotein translocase.[113,114] Class III bacteriocins are typically large (>10 kDa), heat-labile proteins.

Enterocins produced by *Enterococcus faecium*, *E. faecalis*, and *E. mundtii* have been described and include the well-characterized enterocins A, AS-48, B, EJ97, L50, P, Q, 1071, enterolysin A, bacteriocin 31, mundticin, and mundticin KS.[66,68,115–120] Some of these enterocins can be readily grouped into one of the bacteriocin classes as defined by Nes et al.[111] but some have unusual structural or genetic characteristics that do not allow grouping into this classification scheme (Table 3).

Class I Enterocins. Cytolysin is the only known enterocin that can be classified as a class I bacteriocin. It consists of two peptides, both of which contain lanthionine residues.[121] Cytolysin has bacteriocin as well as haemolytic activity and thus is active against both eukaryotic cells (erythrocytes) and gram-positive bacteria.[121,122] The genetic locus for cytolysin production is located on the 58 kb pheromone-responsive plasmid (see virulence factors below) pAD1.[122] The two structural subunits are encoded by two open reading frames (ORFs) ($cylL_L$ and $cylL_S$). The preprotein encoded by these

Table 3 Amino Acid Sequence and Molecular Weight of Enterocins from Different *Enterococcus* Species

Enterocin	N-terminal extension	Amino acid sequence of mature peptide	Molecular weight of mature bacteriocin (Da)
Cytolysin ($CylL_{L''}$)	MENLSVVPSFEELSVEEMEAIQGS	TTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC	3437
Cytolysin ($CylL_{S''}$)	VLNKENQENYYSNKLELVGPSFEEL SLEEMEAIQGS	TTPACFTIGLGVGALFSAKFC	2031
Enterocin A	MKHLKILSIKETQLIYGG	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC	4829
Mundticin	n.r.	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK	4287
Mundticin KS	MKKLTAKEMSQVVGG	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS	4287
Enterocin CRL35	n.r.	KYYGNGVTLNKXGXSVNXXXA. . ..	Unknown
Enterocin P	MRKKLFSLALIGIFGLVVTNFGTKVDA	ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH	4493
Bacteriocin 31	MKKKLVICGIIGIGFTALGTNVEA	ATYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR	n.r.
Enterocin B	MQNVKELSTKEMKQIIGG	ENDHRMPNELNRPNNLSKGGAKCGAAIAGGLFGIPKGPLAWAAGLAN VYSKCN	5463
Enterocin Q	None	MNFLKNGIAKWMTGAELQAYKKKYGCLPWEKISC	3950
Enterocin EJ97	None	MLAKIKAMIKKFPNPYTLAAKLTTYEINWYKQQYGRYPWERPVA	5340
Enterocin L50A	None	MGAIAKLVAKFGWPIVKKYYKQIMQFIGEGWAINKIIEWIKKHI	5190
Enterocin L50B	None	MGAIAKLVTKFGWPLIKKFYKQIMQFIGQGWTIDQIEKWLKRH	5178
Enterocin 1071A	MKQYKVLNEKEMKKPIGG	ESVFSKIGNAVGPAAYWILKGLGNMSDVNQADRINRKKH	4284
Enterocin 1071B	MKNIKNASNIKVIEDNELKAITGG	GPGKWLPWLQPAYDFVTGLAKGIGKEGNKNKWKNV	3897
AS-48		MVKENKFSKIFILMALSFLGLALFSASLQFLPIAH MAKEFGIPAAVAGTVLNVVEAGGWVTTIVSILTAVGSGGLSLLAAAG RESIKAYLKKEIKKKGKRAVIAW	n.r.
Enterolysin A	MKNILLSILGVLSIVVSLAFSSYSVNA	ASNEWSWPLGKPYAGRYEEGQQFGNTAFNRGGTYFHDGFDFGSAIYG NGSVYAVHDGKILYAGWDPVGGGSLGAFIVLQAGNTNVIYQEFSRNV GDIKVSTGQTVKKGQLIGKFTSSHLHLGMTKKEWRSAHSSWNKDDGT WFNPIPILQGGSTPTPPNPGPKNFTTNVRYGLRVLGGSWLPEVTNFN NTNDGFAGYPNRQHDMLYIKVDKGQMKYRVHTAQSGWLPWVSKGDKS DTVNGAAGMPGQAIDGVQLNYITPKGEKLSQAYYRSQTTKRSGWLKV SADNGSIPGLDSYAGIFGEPLDRLQIGISQSNPF	34 501

n.r.: not reported.

ORFs are posttranslationally modified to yield the active subunits $CylL_{L''}$ and $CylL_{S''}$ with a molecular mass of 3437.98 and 2031.81 Da, respectively[121,122] (Table 3).

Class II Enterocins

CLASS IIA ENTEROCINS. Enterocin A (EntA) is a class IIa bacteriocin that contains a N-terminal YGNGVXC motif and is active against *L. monocytogenes*, characteristics that allow grouping of this bacteriocin as a class IIa bacteriocin. It is produced by *E. faecium* strains CTC492 and T136 (both isolated from Spanish fermented sausages), as well as strain BFE 900 isolated from black olives.[65,68,123] EntA consists of 47 amino acids with a theoretical molecular weight of 4829 Da. (Table 3) and is produced as a prepeptide bearing an 18-amino-acid leader peptide of the double-glycine type.

Mundticin KS produced by *Enterococcus mundtii* NFRI 7393 also belongs to the class IIa bacteriocins and exhibits activity against *Lactobacillus* spp., *Enterococcus* spp., as well as *L. monocytogenes*.[118] The mature bacteriocin contains 43 amino acids (Table 3) and is produced as a preprotein bearing a 15-amino-acid leader peptide of the double-glycine type.[118] Mundticin KS is identical to the previously described mundticin produced by *E. mundtii* ATO6[124] (see below), except that the last two C-terminal amino acids are inversed in position when comparing the amino acid sequences of mundticin and mundticin KS.[118] Thus, while mundticin contains Ser^{42} Lys^{43}, mundticin KS contains Lys^{42} Ser^{43}.

CLASS IIB ENTEROCINS. Enterocin 1071 (Ent 1071) is produced by *E. faecalis* strains BFE 1071[116] and FAIR-E 309[117] as a two-peptide bacteriocin from the two structural subunits enterocin 1071A and B. This enterocin has antimicrobial activity against a broad range of gram-positive bacteria, including *Clostridium tyrobutyricum*, *E. durans*, *E. faecalis*, *L. salivarius*, *L. innocua*, and *Micrococcus* spp.[116] Chemical and genetical characteristics of enterocin 1071 were studied by Balla et al.[116] and Franz et al.[117] Both bacteriocin subunits are encoded as prepeptides, each bearing a double-glycine-type leader peptide of either 18 amino acids (Ent1071A) or 24 amino acids (Ent 1071B).[117] The molecular mass of the purified bacteriocins is 4285 and 3899 Da for enterocins 1071A and 1071B, respectively.[116] The genetic determinants for this two-peptide bacteriocin are located on a plasmid DNA,[116,117] and DNA sequencing revealed two ORFs encoding the 39- and 34-amino-acid mature enterocin 1071A and 1071B peptides, respectively (Table 3). The two peptides have homology only to the α and β peptides of the two-component bacteriocin lactococcin G.[117]

Enterocin L50 (Ent L50) is produced by *E. faecium* strains L50 and 6T1a.[99,125] Enterocin L50 consists of two structural subunits, peptides L50A and L50B, both of which have antimicrobial activity and which exhibit synergism when combined. The genetic locus for enterocin L50 was cloned and sequenced and contained the EntL50A and B structural genes encoding the 44-amino-acid (EntL50A) and 43-amino-acid (EntL50B) peptides, which have theoretical molecular weights of 5190 and 5178 Da (Table 3), respectively.[125] The A and B peptides have 31-amino-acid residues in common, and they are 72% identical. Enterocin L50 exhibits a wide antimicrobial spectrum, and it is active against strains of *Enterococcus*, *Lactobacillus*, *Lactococcus lactis*, *Pediococcus pentosaceus*, as well as the foodborne pathogens *L. monocytogenes* and *B. cereus*,[99,125] EntL50A and B are different from other class II bacteriocins, however, as the structural genes do not encode prepeptides but encode the mature bacteriocin without an N-terminal extension.[125] Cintas et al.[125] suggested that although enterocin L50 may be considered a class IIb bacteriocin according to the classification systems of Nes et al.[111] it has

more in common with a group of staphylococcal peptide toxins, which include δ-lysin, SLUSH A–C and AGS1–3 produced by *Staphylococcus aureus*, *S. lugdunensis*, and *S. haemolyticus*.

CLASS IIC ENTEROCINS. Enterocin P (EntP) is produced by *E. faecium* strains P13 and L50, both isolated from Spanish fermented sausages.[66,115] Its antimicrobial spectrum includes activity against *Enterococcus*, *Lactobacillus*, and *Pediococcus* spp., and *B. cereus*, *S. aureus*, *Clostridium perfringens*, *C. botulinum*, and *L. monocytogenes*.[66] The mature enterocin P bacteriocin consists of 44 amino acids and has a theoretical molecular weight of 4493 Da (Table 3). Enterocin P is produced as a prepeptide of 71 amino acids with a 27-amino-acid signal peptide (Table 3). Secretion of enterocin P, therefore, occurs by the bacterial preprotein translocase,[66] which is a determinative characteristic for grouping this bacteriocin into class IIc bacteriocins according to the classification scheme of Nes et al.[111] Enterocin P has sequence similarity with the other class II bacteriocins, such as sakacin A, carnobacteriocins BM1 and B2, leucocin A, mesentericin Y105, and sakacin P.[66]

Bacteriocin 31 is produced by *E. faecalis* YI717, and the genetic determinants involved in production for this bacteriocin are located on a pheromone-responsive plasmid.[126] Bacteriocin 31 is also secreted by the bacterial preprotein translocase and contains a 24-amino-acid signal peptide.[126] Alignment of the bacteriocin 31 and enterocin P signal peptide amino acid sequences showed that they share sequence identity of 12 amino acids.[66] The mature bacteriocin 31 consists of 43 amino acids (Table 3), and it is active against *Enterococcus* spp. and *L. monocytogenes*.

OTHER CLASS II ENTEROCINS. Mundticin, produced by an *E. mundtii* strain isolated from processed vegetables[124] consists of 43 amino acids and contains a YGNGVXC-consensus motif at the N-terminus of the bacteriocin molecule. Mundticin has a molecular weight of 4287 Da as determined by mass spectrometry and has activity against *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus* spp., as well as *L. monocytogenes* and *C. botulinum*. The genetics of mundticin production have not been studied, and it is thus not known whether this bacteriocin is secreted by dedicated transport proteins or by the bacterial pre protein translocase[111]. For this reason, it is not clear whether mundticin belongs to the class IIa or class IIc bacteriocins as defined by Nes et al.[111].

Enterocin CRL35 produced by *E. faecium* CRL35 shows strong sequence homology to other class II bacteriocins, leucocin A, curvacin A, and sakacins A and P. It also contains a YGNGVXC motif near the N-terminus (Table 3); however, the characteristic cysteine that is located two amino acid residues from the valine is not present. Instead, this bacteriocin has a YGNGVXL motif at the N-terminus.[127] The full amino acid sequence of this bacteriocin has not been determined. The sequence reported by Farías et al.[127] has strong sequence similarity at the N-terminus with mundticin (Table 3). The gene encoding the prepeptide of enterocin CRL35 has not been cloned and sequenced, therefore it is not yet clear whether this bacteriocin belongs to the class IIa or class IIc bacteriocins.

Class III Enterocins. Enterolysin A is a bacteriocin produced by *E. faecalis* LMG 2333. It is a large (calculated molecular weight of 34,501 Da), heat-labile bacteriocin[128] and therefore fits the general characteristics of class III bacteriocins. Enterolysin A is inhibitory towards *Enterococcus*, *Pediococcus*, *Lactococcus*, and *Lactobacillus* spp. The genetic determinant for enterolysin A was cloned and sequenced.[128] The bacteriocin is encoded as a prepeptide consisting of a 316-amino-acid protein bearing a 27-amino-acid signal

peptide. Amino acid sequence comparison indicated homology to cell wall–degrading proteins such as lysostaphin produced by *Staphylococcus simulans* biovar. *staphylolyticus* and to ALE-1, LytM, and zoocin A, which are all endopeptidases belonging to the M37 protease family.[128]

Atypical Enterocins. Enterocin B (Ent B) is produced in addition to EntA by *E. faecium* strains CTC492, T136, and BFE 900.[12,65,68] Enterocin B does not contain the YGNGVXC-consensus motif at the N-terminus of the mature peptide (Table 2), which makes it different from the class IIa and class IIb bacteriocins. Enterocin B shares sequence similarity only with carnobacteriocin A,[12,129] which also does not contain the YGNGVXC-consensus motif. Enterocin B is active against *Enterococcus* and *Lactobacillus* spp. and the foodborne pathogens *L. monocytogenes*, *S. aureus*, and *C. perfringens*.[12,65] The bacteriocin consists of 53 amino acids with a molecular weight of 5463 Da (Table 3). Genetic analysis showed that the prepeptide contained an 18-amino-acid leader peptide of the double-glycine type.[12,65] This indicated that EntB was secreted by a dedicated-type secretion mechanism, involving the ABC transporter and accessory proteins. However, a 12.0 kb DNA fragment cloned from the chromosome of *E. faecium* BFE 900 that contained the structural gene for EntB did not contain ABC transporter or accessory protein genes.[12] It is possible that EntB is secreted by the dedicated transport proteins of EntA or some other transport system available in the cell.[12]

Enterocin Q (EntQ) is produced by *E. faecium* L50 in addition to the bacteriocins enterocin P and enterocin L50.[114] Enterocin Q is a unique bacteriocin, as it has a relatively small size of 34 amino acids (Table 3) with a theoretical molecular mass of 3952 Da[115] and, like Enterocin L50, is also not produced as a preprotein. This feature (absence of a N-terminal extension involved in bacteriocin transport) actually makes both the enterocin L50 and enterocin Q atypical enterocins, which cannot be grouped into any of the class II bacteriocin subgroups. Cintas et al.[115] suggested that enterocin Q is transported from the cell by a presently undetected ABC transporter, as has been demonstrated for other bacterial proteins that do not contain an N-terminal leader or signal peptide. Enterocin Q also lacks the YGNGVXC consensus sequence at the N-terminus.

Enterocin EJ97 (Ent EJ97) is a bacteriocin produced by *E. faecalis* EJ97, which is inhibitory to species of *Bacillus*, *Enterococcus*, *Listeria*, and *Staphylococcus*.[130] The genes for enterocin EJ97 production were detected on a 60 kb conjugative, pheromone-response plasmid pEJ97, and the structural gene encodes a 44-amino-acid bacteriocin (Table 3) that, similar to EntL50 and EntQ, does not contain a N-terminal extension.[120] Again in this case, an assignment of this enterocin into one of the class II subclasses, due to the lack of a leader or signal peptide, is problematic. Genes encoding putative transport proteins such as an ABC transporter and a putative accessory protein were found in close proximity of the Ent EJ97 structural genes and may be involved in transport and possibly immunity.[120] A search of the protein databanks did not reveal homology to any of the bacteriocins described to date.[120]

Enterocin AS-48 is produced by a clinical isolate of *E. faecalis* strain S-48.[131] Production of identical bacteriocins, bacteriocin 21 and enterocin 4 was subsequently reported for other strains of *E. faecalis*.[132,133] The structural genes for enterocin AS-48 are located on a pheromone-responsive plasmid, and Martínez-Bueno et al.[134] showed that the *as-48* gene encoded a 105-amino-acid prepeptide consisting of a 35-amino-acid signal peptide and a 70-amino-acid mature peptide (Table 3). AS-48 forms a 70-amino-acid cyclic molecule that results from a head-tail linkage of the N-terminal

methionine (M^{+1}) to the C-terminal tryptophan (W^{+70}).[134] Thus, the cyclic nature of AS-48 makes this bacteriocin molecule quite unique when compared to the linear bacteriocins, which are otherwise generally produced by LAB.

As shown above, enterocins produce an impressive array of antimicrobial substances, which may, in part, also explain the success of these bacteria to successfully compete in such various niches such as the gastrointestinal tract and various foods, in which they are found together with a complex associative microflora. Bacteriocin activity of enterococci against foodborne pathogens, especially *L. monocytogenes*, and food-spoilage bacteria such as *Clostridium tyrobutyricum*, which is involved in the "blowing" defect of cheeses, has stimulated interest in using these bacteria or their purified bacteriocins for use in food production as biopreservatives.

2. Use of Enterocins or Enterocin-Producing Enterococci in Biopreservation

Bacteriocin production by enterococci isolated from dairy products has been investigated. Strains producing enterocin AS-48[131,134,135] have been found in raw milk and dairy products.[136–139] *E. faecium* WHE 81 isolated from cheese produces enterocins A and B that also inhibit *L. monocytogenes*.[140,141] Another strain, *E. faecium* EFM01, also isolated from cheese, was shown to produce enterocin A.[142] Similarly, strains producing the enterocins A, B, P, L50, and Q were isolated from certain types of fermented sausages, particularly the dry fermented sausages known as chorizo[65–68,143] and espetec[68] produced in Spain. Production of antilisterial bacteriocins by various enterococci from Spanish-style dry fermented sausages were proposed to make these suitable for addition to meat as co-cultures to improve food safety.[65,115,144]

Enterocins or starter cultures containing bacteriocin-producing enterococci have been used in model studies to improve safety of the cheeses.[93,145–150] A bacteriocinogenic *E. faecium* strain was tested on a laboratory scale for use in combination with a commercial starter culture for Taleggio cheese making.[106] The bacteriocin was produced during drainage of the whey, and activity could be detected in the cheese until the end of the ripening period, while growth and acidifying activity of the thermophilic commercial starter culture was not inhibited.[106] An inhibitory starter culture consisting of bacteriocinogenic *E. faecium*, *E. faecalis*, nisin-producing *L. lactis* and *Lactobacillus paracasei* added to milk prior to Camembert cheese making or sprayed onto the surface of the cheese, totally inhibited *Listeria* spp. when surfaces were contaminated with *Listeria* not later than 1.5 days after brining.[150] Lauková and Czikková[146] added the semi-purified bacteriocin enterocin CCM 4231 to bryndza cheese that was experimentally contaminated with *L. innocua*. Only a slight reduction ($\sim$1 log unit) was noted when comparing this cheese to a control inoculated with *L. innocua* at a similar level, but to which no bacteriocin was added.[146] Garcia et al.[145] used an *E. faecalis* strain INIA4 which produces AS-48 to study inhibition of a mixture of *L. innocua* strains during manchego cheese making. The effect of this bacteriocin appeared to be mostly bacteriostatic during the first 24 hours of cheese making, when pH was not low enough to inhibit *L. innocua* growth. However, the decrease in *L. innocua* counts during the ripening of the cheese appeared to be mostly due to the low cheese pH values.[145] Sarantinopoulous et al.[93] used a bacteriocin-producing *E. faecium* FAIR-E 198 strain as adjunct starter culture in feta cheese-making; no enterocin activity could be detected during cheese ripening.

Thus, varying levels of success were achieved and it was suggested that rennet, $CaCl_2$, and non-*Enterococcus* starter cultures may influence bacteriocin production and

hence the successful inhibition of target bacteria.[93,145,149] Thus in vitro production of bacteriocins is no guarantee for in situ inhibitory efficiency, as the complex food constituents may interfere with bacteriocin production levels.[93]

Enterocin-producing strains or purified enterocins have also been used for biopreservation of meats in model studies. Enterocins A and B from *E. faecium* CTC492, when added as semi-pure preparations, showed a marked antilisterial activity in model meat and meat products such as cooked ham, minced pork meat, deboned chicken breasts, pâté, and espetec. The bacteriocin-producing strain itself was not used as a starter culture, because bacteriocin production and growth of the strain were inhibited by low temperatures and the salt and pepper ingredients used in the sausage recipe.[151] In contrast, Callewaert et al.[144] showed that two bacteriocin-producing strains of *E. faecium* effectively inhibited a strain of *L. innocua* in model Spanish-style dry fermented sausage. Therefore, it was suggested that particular enterocins could be considered as additional biopreservative hurdles for successful prevention of listerial growth in fermented sausages.[144,151,152]

B. Other Technological Traits of Enterococci for Use as Cheese Starter Cultures

Because of their role in ripening and flavor development in cheeses, enterococci with desirable technological and metabolic traits have been proposed as part of defined starter cultures or as adjunct starter cultures for different European cheeses.[90,153–155] The ability of enterococci to grow at low pH and high salt concentration and their relative heat resistance[85,94] are traits that enable enterococci to occur in and grow in cheeses and meats, and as such these may already be considered as technological traits.

1. Acidifying Activity

As members of the lactic acid bacteria, enterococci produce lactic acid as end product of metabolism, and this acidifying activity, which is important for any food fermentation, can be considered as an additional technological trait. However, in milk and in meats the enterococci generally exhibit only low acidifying ability.[151,152,156] Morea et al.[157] showed that the pH of milk 24 hours after inoculation with enterococci strains isolated from mozzarella cheese did not decrease below pH 5.5. Other investigations[34,158] confirmed the poor acidifying activity of enterococci in cheese production as only a small percentage of strains could decrease the pH below 5.0–5.2 after 16–24 hours of incubation at 37°C.[156] Delgado et al.[88] showed that some enterococci isolated from cheese produced acid at sufficient levels for production of artisanal cheeses. Two *E. durans* isolates in particular were able to reduce the pH of skim milk below pH 4.4 after 24 hours at 22°C.[88] *E. faecalis* generally appears to be a stronger acidifier than *E. faecium*, and lowering of skim milk pH to about pH 4.5 after 24-hour fermentation was observed for *E. faecalis* strains isolated from Italian cheeses.[156,159]

2. Proteolytic, Lipolytic, and Esterase Activity

Proteolytic activity of enterococci for breakdown of milk casein is important for cheese ripening. Conflicting reports on proteolytic activity of enterococci suggest a marked strain-to-strain variation of this phenotypic trait.[88,156,160,161] Although there are exceptions, generally the proteolytic activity of enterococci appears to be low, with *E. faecalis* strains possessing higher activity than other *Enterococcus* species.[34,89,90,156,162,163]

Esterases are arbitrarily defined as enzymes that hydrolyze substrates in solution, while lipases hydrolyze substrates in emulsion.[156] Esterases have been linked to the flavor development and cheese texture by lipolysis of milk fat and subsequent conversion of the free fatty acids produced to methylketones and thioesters, which have importance as cheese flavor compounds. Lipolysis, on the other hand, is not directly involved in cheese rheology but partial glycerides are tensio-active and influence molecular organisation, thus having an effect on cheese texture.[156] Hydrolysis of triglycerides by enterococci has been reported, with *E. faecalis* strains appearing to be most active,[161–164] while the esterolytic system of enterococci appears to be complex and more efficient than their lipolytic system.[156] *E. faecium* strains appear to be more esterolytic than other enterococci species.[95,156,162,163]

3. Citrate Metabolism

Citrate metabolism plays an important role in many food fermentations involving lactic acid bacteria, as this compound occurs in many natural food substrates such as milk, vegetables, and fruits. Since citrate is a highly oxidized substrate, no reducing equivalents, such as NADH, are produced during its degradation, which results in the formation of metabolic end products other than lactic acid.[162] Some of these end products, such as diacetyl, acetaldehyde, and acetoin, have distinct aroma properties and can significantly influence the quality of fermented foods. In addition, the breakdown of citrate also results in the production of CO_2, which can contribute to the texture of some fermented foods.[162] Freitas et al.[87] indicated that citrate in milk was metabolized by *E. faecalis* and to a lesser extend by *E. faecium*. Sarantinopoulos et al.[158] showed that strains of *E. faecalis*, *E. faecium*, and *E. durans* varied in their ability to utilize citrate or pyruvate as the sole carbon sources. Sarantinopoulous et al.[162] showed that in skim milk citrate and lactose were co-metabolized by *E. faecalis* FAIR-E 229, while in MRS broth containing citrate and lactose or glucose as the sole carbohydrate, citrate was not catabolized but stimulated the growth of the *E. faecalis* strain. When present as a sole carbon source in MRS broth without glucose, citrate was catabolized by *E. faecalis* FAIR-E 229 with the main end products being acetate and formate, while minor amounts of lactate, ethanol, and acetoin were also detected. The work of Sarantinopoulous et al.[162] indicated that *Enterococcus* strains have the metabolic potential to metabolise citrate and thus actively contribute to flavor development of fermented dairy products.

As shown above, enterococci are not good acidifiers of milk and meats, and their proteolytic and esterolytic properties may not be high. For this reason it would probably be better to use enterococci in food fermentations as adjunct starter cultures in combination with established starter strains, rather than using these bacteria as defined starter cultures by themselves. Nevertheless, the effect of the technological properties of the enterococci is not negligible and should not be underestimated. For example, Sarantinopoulous et al.[93] studied the technological properties of two strains of *E. faecium* as adjunct starter cultures, either single or combined, on the microbiological, physicochemical, and sensory characteristics of feta cheese in a well-defined study. It was shown that the presence of the enterococcal starter strains positively affected the growth of nonstarter LAB, increased the proteolytic index and free amino group concentration, enhanced the water-soluble nitrogen fractions, and positively affected taste, aroma, color, structure, and the overall sensory profile of the cheese.[93] Clearly, the results of these study supported previous suggestions that enterococci indeed positively influence cheese fermentations.

C. Production of Biogenic Amines During Fermentation

Numerous bacteria, including strains of LAB, associated with food fermentation are able to form biogenic amines (BA). As a result of high metabolic activities, amino acid decarboxylase positive strains may produce relatively high amounts of BA, especially in protein-rich substrates such as meat and milk. Ingestion of high levels of biogenic amines such as histamine and tyramine may result in allergic reactions or cause intoxication symptoms such as headache, vomiting, and increased blood pressure. Within the LAB, amino acid (and particularly tyrosine) decarboxylase activity appears to be quite common among be the enterococci. Their ability to produce BA, and especially tyramine, has been reported particularly for cheese and fermented sausages.[85,165–167] Within 25–37°C and pH 5–7, the production conditions for BA-positive enterococci appear to be optimal. Contrary to the general expectation for BA-positive LAB, the tyrosine decarboxylase activity of enterococci was reported to increase with increasing NaCl concentrations of up to 10% (and with elevated production levels even at 20% NaCl), concomitantly with an increased production rate. In addition, it has been shown that levels of 1–2% $NaNO_2$ also result in increased production of tyramine in a model system with a strain of *E. faecalis*.[168] The wide distribution and association of enterococci with traditional fermented foods may to some extent explain the elevated levels of tyramine that may be found in these products.

D. Enterococci as Probiotics

Functional effects claimed for probiotics include inhibition of pathogenic microorganisms, strengthening of the gut mucosal barrier, antimutagenic and anticarcinogenic activities, stimulation of the immune system, and lowering of blood cholesterol levels.[169–174] Most probiotic cultures are of intestinal origin and belong to the genera *Bifidobacterium* and *Lactobacillus*; however, *Enterococcus* spp. are also occasionally used as probiotics.

E. faecium SF68 has been used to treat diarrhea and is considered as an alternative to antibiotic treatment.[175,176] Several placebo-controlled, double-blind clinical studies have shown that treatment of enteritis with *E. faecium* SF68 was successful for both adults and children. It decreased the duration of diarrheal symptoms and the time for normalization of patient's stools.[175–178] The use of *E. faecium* SF68 as an animal probiotic was also investigated. Vahjen et al.[179] showed that turkey poults fed the probiotic showed a continuous increase in lactate concentrations in the small intestine, which in turn stimulated the growth of other LAB, especially lactobacilli. This stimulatory effect of lactobacilli was suggested to benefit the animal as it could repress potentially harmful bacteria during the early stages of life.[179] Benyacoub et al.[180] studied the effect of the probiotic *E. faecium* SF68 on young dogs and found that the probiotic enhanced specific immune functions when compared to an untreated control group. Administering the probiotic occurred at the time at which a vaccine for canine distemper virus was given. It could be shown that fecal IgA and canine distemper vaccine–specific circulating IgG and IgA were higher in the treated puppy group when compared to the control group Furthermore, mature B cells ($CD21+/MHC\ class\ II^+$) were greater in the group fed the probiotic.[180]

Another probiotic for human use that contains enterococci is the Causido® culture that consists of two strains of *S. thermophilus* and one of *E. faecium*. This probiotic has been claimed to be hypocholesterolaemic in the short term,[181] but long-term reduction

of low-density lipoprotein (LDL) cholesterol levels was not demonstrated;[182,183] hence, the clinical relevance of this effect is uncertain.[184]

Cholesterol-lowering effects of probiotics have often been linked to bacterial bile salt hydrolase (BSH) activity. BSH activity is mediated by various gram-positive intestinal bacteria, including members of the genera *Enterococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Clostridium*, *Bacteroides*, and *Lactobacillus*.[185,186] The conjugated BSH enzyme (E.C. 3.5.1.24) deconjugates bile salts by liberating the glycine and/or taurine moiety from the side chain of the steroid core. It was hypothesized that deconjugation of bile salts may contribute to lower cholesterol levels because free bile acids may more likely be excreted from the gastrointestinal tract than conjugated bile salts.[186] If enhanced fecal loss of bile acids occurs as a result of bacterial BSH activity, it may increase the demand for cholesterol as a precursor for de novo synthesis of bile salts, which in turn may lower cholesterol levels.[187,188] In a study on enterococci isolated from foods, we screened 117 *Enterococcus* strains isolated from food (47 *E. faecium*, 48 *E. faecalis*, 16 *E. durans*, 2 *E. gallinarum*, 3 *E. casseliflavus*, and 1 *E. malodoratus*) for BSH activity. The highest incidence of BSH active strains was observed for *E. faecalis* (81%) followed by *E. faecium* (50%) and *E. durans* (44%). Thus, bile salt hydrolase activity appears to be a fairly common trait among enterococci. In addition to its suggested effects of lowering cholesterol levels in probiotic strains, BSH activity was also suggested to allow bacteria to survive conditions and grow in the gastrointestinal tract.[189] For a probiotic strain, this could be interpreted as a beneficial technological trait. In contrast to the desirable cholesterol-lowering effect, it should be mentioned that BSH activity may also have adverse effects in that extensive deconjugation of bile salts in the human small bowel can lead to steatorrhea and that secondary (dehydroxylated) bile salts, generated by the enzymatic conversion of primary bile salts by other intestinal bacteria, are cytotoxic and co-carcinogenic.[190]

The use of enterococci as probiotics remains a controversial issue. While the probiotic benefits of some strains are well established, the emergence of antibiotic-resistant strains of enterococci and the increased association of enterococci with human disease (see below) have raised concern regarding their use as probiotics. The fear that antimicrobial resistance genes or genes encoding virulence factors can be transferred to probiotic strains in the gastrointestinal tract contributes to this controversy.

VI. ENTEROCOCCI IN HUMAN DISEASE

A. Infections Caused by Enterococci

Enterococci are typical opportunistic pathogens and usually cause infections in patients who have severe underlying disease, who have received surgery, or who are immunocompromised.[191] They are usually associated with hospital-acquired infections and cause bacteremia, endocarditis, and urinary tract and other infections.[191,192] They rank among the most prevalent organisms encountered in nosocomial infections, accounting for approximately 12% of nosocomial infections in the United States.[193] *E. faecalis* predominates among enterococci isolated from human infections (>80%), while *E. faecium* is associated with the majority of the remaining infections.[194] A shift towards *E. faecium* strains as the causative agent in enterococcal bacteraemia was noted, probably because of the emergence of vancomycin-resistant strains.[195]

Bacteremia is a common form of opportunistic enterococcal infection.[191,196,197] Compared with a steady reduction in community-acquired cases of enterococcal bacteremia, nosocomial cases may have increased threefold and account for up to 77% of cases.[191,198–200] Risk factors associated with enterococcal bacteremia include underlying disease, presence of urethral or intravascular catheters, surgery, major burns, multiple trauma, or prior antibiotic therapy.[196] Sources of enterococci causing bacteraemia without endocarditis are most commonly from the urinary tract, but the gastrointestinal and hepatobiliary tracts have also been implicated.[43,190,196] Mortality from enterococcal bacteremia is generally high, most probably because of the underlying complicating factors.[192,201]

Enterococci cause an estimated 5–15% of cases of bacterial endocarditis, with *E. faecalis* more commonly involved than *E. faecium*.[191,192] The enterococci usually originate from the urinary tract,[43,194,200] and underlying heart disease is often present, but it is not a prerequisite for development of this infection.[43,191,192] Endocarditis often occurs in patients that had preceding genitourinary instrumentation or urinary tract infection (UTI), abortion, or urinary tract instrumentation.[43,192,203]

Urinary tract infections are commonly caused by enterococci, especially in hospitalized patients. These infections occur especially in persons who had surgery, received antibiotics, had structural abnormalities, or had recurrent enterococcal infections.[43,192,203]

Infections of the central nervous system by enterococci are rare and are seen primarily in neonates and persons who have undergone complicated neurological procedures.[191,203] Enterococci causing neonatal infection are thought to originate from the vagina, because they are detected in the vaginal microflora in 25% of healthy women.[196] *E. faecium* and *E. faecalis* have been implicated in outbreaks of neonatal central nervous system infections, although infections of older children and adults have also been reported.[192,204]

Enterococci may cause or contribute to abdominal and pelvic abscess formation and sepsis.[192] They were reported as a cause of spontaneous peritonitis in cirrhotics and nephrotics and may be associated with peritonitis in patients on peritoneal dialysis.[19,192] Dialysis catheters and prior use of antibiotics are predisposing factors for intra-abdominal infections by enterococci.[43,205]

B. Antibiotic Resistance

A specific cause for concern and a contributing factor to pathogenesis of enterococci is their resistance to a wide variety of antibiotics.[192,206,207] Enterococci are either intrinsically resistant and resistance genes are located on the chromosome, or they possess acquired resistance determinants which are located on plasmids or transposons.[192,208] Examples of intrinsic antibiotic resistance include resistance to cephalosporins, β-lactams, sulfonamides, and low levels of clindamycin and aminoglycosides, while examples of acquired resistance include resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, high levels of β-lactams, fluoroquinolones, and glycopeptides such as vancomycin.[192,207]

Intrinsic resistance to many antibiotics suggests that treatment of infection could be difficult. However, combinations of cell-wall–active antibiotics such as penicillin or ampicillin with aminoglycosides (e.g., streptomycin, kanamycin, and gentamicin) act synergistically and have been used successfully in treatment of enterococcal infection.[192,209–211] Since the early 1970s, a high level of streptomycin and gentamicin resistance was reported, and strains resistant to penicillin-streptomycin or penicillin-gentamicin

combinations were also found.[209] In 1983 a strain of *E. faecalis* producing a β-lactamase identical to that produced by *S. aureus* was reported, and it is believed that this strain of *Enterococcus* received the gene from *S. aureus*.[212] The hitherto successful penicillin-aminoglycoside treatment was no longer a viable option, resulting in a major therapeutic problem.[191,210]

Enterococcal penicillin resistance occurs by two different mechanisms. One involves the production of penicillin-binding proteins (PBPs) with decreased affinity for the antibiotic, while the second involves hydrolysis of the penicillin molecule by β-lactamase. While resistance based on PBPs with reduced affinity for penicillin is more common among *E. faecium* strains, resistance based on β-lactamase is more common for *E. faecalis* strains.[213]

The gene encoding high level resistance (HLR) to gentamicin in *E. faecalis* has the same nucleotide sequence as the gentamicin resistance gene of staphylococci, and it was suggested that enterococci received this gene from staphylococci.[214,215] HLR to gentamicin in *E. faecium* occurred after its appearance in *E. faecalis* and was first reported in 1998.[215] Acquired high-level gentamicin resistance results from the transfer of genes encoding aminoglycoside-modifying enzymes by conjugative plasmids and transposons.[211] HLR to aminoglycosides in enterococci is due to the synthesis of one or more of a series of aminoglycoside-modifying enzymes such as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferase (AAD), and aminoglycoside phosphotransferases (APH). The aminoglycoside-modifying enzymes that have been identified in enterococci (and staphylococci) are APH(3′), APH (2″), AAD(6), AAD(4′), and AAC(6′).[211] Ferretti et al.[214] showed that the genes for the two aminoglycoside-modifying enzymes aac-6′ and aph-2″ had probably fused to generate an extremely powerful enzyme complex conferring resistance to all clinically useful aminoglycosides (gentamicin, tobramycin, amikacin, and netilmicin) with the exception of streptomycin. The worldwide dissemination of HLR to gentamicin appears to stem from the spread of this enzyme's (aac6′-aph2″) gene via plasmids and transposons.[211]

Vancomycin resistance is of special concern because this antibiotic was considered a last resort for treatment of multiply resistant enterococcal infections. In addition, this antibiotic was given as an alternative to ampicillin or penicillin/aminoglycoside treatment to persons with allergy against penicillin or ampicillin.[191] In the mid-1990s in Europe the source of vancomycin-resistant enterococci (VRE) was shown to be most likely farm animals as a result of ergotropic use of avoparcin, a glycopeptide antibiotic.[216–218] VRE have indeed been isolated from a wide variety of farm animals, and these constitute an important reservoir of VRE that could be transmitted to the hospital environment via contaminated meat.[216,217,219–221] These findings strongly suggest that food transmission occurred, and, as a result, two European countries (Denmark and Germany) banned the use of avoparcin,[191] followed by a European Union–wide ban.[222] Not only food, but also contact between the farm animal and humans at avoparcin-exposed farms also resulted in human VRE colonization.[223,224] There are conflicting results on the effect of the avoparcin ban on the incidence of vancomycin resistance among enterococci. Borgen et al.[55,225] showed that VRE were isolated from poultry and poultry farmer's feces as well as poultry carcasses at a high incidence even 3 years after agricultural use of avoparcin was banned. In contrast, Klare et al.[226] showed that the incidence of VRE from frozen and fresh poultry meats decreased 2 years after the avoparcin ban. Moreover, the incidence of VRE isolated from the feces of healthy volunteers had decreased by half.[226]

In the United States, the situation with respect to nosocomial VRE infections appears to differ considerably from that in Europe because avoparcin has not been licensed for use.[222] A community prevalence survey failed to isolate VRE from healthy volunteers without hospital exposure and from environmental sources or probiotic preparations.[227] In contrast to Europe, transmission of VRE in the United States does not appear to be from the community to the hospital, and food has not been implicated as a possible vehicle for transmission. This indicates that clinical use of vancomycin is responsible for development of VRE.

The emergence of vancomycin-resistant enterococci (VRE) in hospitals has led to infections that cannot be treated with conventional antibiotic therapy, and thus such strains pose a serious medical concern.

C. Virulence Factors

Antibiotic resistance alone cannot explain the virulence of enterococci, and to cause infection, enterococci must have virulence factors that allow the infecting strains to colonize host tissue, invade host tissue, and translocate through epithelial cells and evade the host's immune response. Furthermore, such virulent strains must produce pathological changes either directly by toxin production or indirectly by inflammation.[228] In the past, enterococci were considered to possess subtle virulence traits that were not easily identified.[194] However, considerable progress has been made in determining virulence traits from clinical isolates, and each of these may be associated with one or more of the stages of infection mentioned above.

1. Aggregation Substance

Aggregation substance (AS) (Table 4) is an adhesin that is encoded on pheromone-responsive plasmids. Expression of the AS gene is induced by sex pheromones that are small (7–8 amino acids) hydrophobic peptides, which are excreted by plasmidless, recipient strains of *E. faecalis*. Binding of the pheromones by the donor strain leads to expression of AS on the cell surface. AS leads to clumping of donor and recipient cells by binding to a complementary receptor termed binding substance. The clumping of cells leads to a highly efficient transfer of the pheromone plasmid on which the AS gene is encoded.[229,230]

However, AS is also involved in binding to eukaryotic cells. The molecule contains two RGD (Arg-Gly-Asp) amino acid motifs that promote *E. faecalis* adhesion to eukaryotic cells, such as pig renal tubular cells, via integrin receptors.[231] AS was shown to bind to a variety of cells via such β_2-type integrins, including human macrophages and intestinal epithelial cells.[232,233] Moreover, AS was determined not only to bind to eukaryotic cells, but also to extracellular matrix (ECM) proteins such as fibronectin, thrombospondin, vitronectin, and collagen type I.[234] To cause abdominal infection and bacteremia, enterococci must penetrate the intestinal or genitourinary epithelium and enter the lymphatic and/or vascular system.[235,236] During this translocation process, the enterococci encounter the basal membrane and extracellular matrix proteins. Especially in cases of intestinal lesions, the ability to adhere to exposed extracellular matrix proteins is thought to promote bacterial translocation. Only limited data are available on translocation of enterococci through intact epithelial cell layers. Sartingen et al.[232] showed that AS promoted internalization of enterococci by enterocytes derived from the colon and the duodenum, but these AS-producing enterococci strains were not able to translocate enterocytes in an

Table 4 Virulence Factors Found in Some *Enterocccus* Strains and (Suggested) Association with Stage of Virulence

Virulence determinant	(Suggested) association with stage of virulence
Aggregation substance (AS)	Adhesion to eukaryotic cells (adhesin)/ promotes colonization Invasion of eukaryotic cells (invasin) Adhesion to extracellular matrix proteins (may promote translocation) Increases survival in immune cells (evasion of host immune response)
Cytolysin (Cyl)	Eukaryotic cell toxin Lyses immune cells (evasion of host immune response)
Gelatinase (Gel)	Can hydrolyze various biological peptides, e.g., collagens and fibrin (role in translocation?) Can hydrolyze antibacterial peptides (evasion of host innate immune response)
Enterococcal surface protein (Esp_{fs} and Esp_{fm})	Adhesin, promotes colonization Exhibits characteristics of MSCRAMMs—role in evasion of immune response?
Adhesin to collagen of *E. faecalis* (Ace) or *E. faecium* (Acm)	Adhesion to extracellular matrix proteins (may promote translocation) Exhibits MSCRAMM characteristics—role in evasion of immune response?
Endocarditis antigen from *E. faecalis* or *E. faecium* ($EfaA_{fs}$)	Adhesin: role in endocarditis
Hyaluronidase	Degrades hyaluronic acid, a major extracellular matrix constituent—role in translocation?
Capsule	Evasion of host immune response

8-hour period. However, mouse in vivo studies demonstrated that enterococci migrated across the intact intestinal mucosa and spread to the mesenteric lymph nodes, liver, and spleen when intestinal overgrowth with *E. faecalis* was induced.[237] Thus, Sartingen et al.[232] concluded that the incubation time (8 h) to allow for translocation studies may have been too short in their in vitro study. To date, there is no knowledge of virulence factors other than AS which play a role in invasion or translocation of enterococci.

Thus, AS not only functions as an adhesin, but it may also be associated with translocation of enterococci (Table 4). Adherence to ECM proteins is also thought to play a major role in wound infections and in bacterial endocarditis.[234,238] About half of enterococcal endocarditis cases occur where subendothelial extracellular matrix proteins such as fibronectin, collagen, laminin, thrombospondin, and vitronectin are exposed.[234]

AS was shown to facilitate not only adherence, but also invasion of eukaryotic cells in tissue culture.[231,232,239,240] It promoted adherence to human neutrophils (PMNs) as well as adherence to and phagocytosis by human macrophages.[233,241] AS also promoted adherence to and increased intracellular survival of enterococci in host immune cells, such as macrophages and neutrophils (PMNs).[233,242] In activated human neutrophils, it was shown that the phagosomes containing AS-bearing enterococci were markedly larger

than phagosomes containing opsonized *E. faecalis*, suggesting that some modification of phagosomal maturation may be involved in AS-induced resistance to killing. In addition, the pH of PMN phagosomes was significantly higher after ingestion of nonopsonized, AS-bearing *E. faecalis* than after ingestion of opsonized cells.[242] Süssmuth et al.[233] showed that AS-bearing enterococci were significantly more resistant to killing by human macrophage during the first 3 hours postinfection, probably due to inhibition of the respiratory burst.

AS is therefore considered an important multifunction virulence factor because it acts as an adhesin and invasin; in addition, it is involved in translocation as well as evasion of the immune response by intracellular survival in immune cells (Table 4).

2. Sex Pheromones

Sex pheromones themselves can be considered as virulence determinants (Table 4). They are cleavage products of 21- to 22-amino-acid signal peptides associated with surface lipoproteins of unknown function.[243] These, as well as their surface exclusion proteins, are involved in causing pathological changes such as acute inflammation.[228] They are chemotactic for human and rat PMNs in vitro and induce superoxide production and secretion of lysosomal enzymes.[228,244,245]

3. Cytolysin

The β-hemolysin/bacteriocin or cytolysin is a cellular toxin that enhances virulence in animal models.[122,246–248] In Japan, it has been shown that 60% of clinical strains involved in parenteral infection had a hemolytic phenotype, compared with only 17% of isolates from the feces of healthy individuals.[249] Similar trends were observed in a study of *E. faecalis* bloodstream isolates in the United States.[250] However, in a European study, only 16% of *E. faecalis* strains isolated from blood exhibited hemolytic activity.[251] Cytolysin production can be considered as a bacterial strategy to evade the host immune response, as Miyazaki and coworkers[252] showed that hemolytic culture supernatants of *E. faecalis* lysed mouse PMNs and macrophages. Thus, cytolysin can evade a host immune response by destroying cells of the immune system. Production of cytolysin appears to be a major risk factor associated with pathogenic enterococci as Huycke et al.[250] determined a fivefold increased risk of death of patients within 3 weeks of bacteremia caused by β-hemolytic enterococci, compared with bacteremia caused by non-β-hemolytic strains.[250]

4. Enterococcus Surface Protein from *E. faecalis* (Esp_{fs}) and *E. faecium* (Esp_{fm})

The enterococcal surface protein (Esp) produced by either *E. faecalis* (Esp_{fs}) or *E. faecium* (Esp_{fm}) is an adhesin (Table 4), and the gene encoding this trait in both *Enterococcus* species appears to be chromosomally encoded. The incidence of Esp_{fs} was shown to be higher among clinical strains of *E. faecalis* than isolates from healthy individuals,[250] indicating a role in pathogenicity. Similarly, Eaton and Gasson[254] found Esp_{fm} to be highly conserved in infection-derived isolates and environmental isolates, but absent in food and commensal isolates, which led them also to suggest a role in pathogenicity.[255] Shankar et al.[256] used an Esp_{fs}^{+} strain and an isogenic mutant in a mouse model of ascending urinary tract infection to show that Esp_{fs} contributed to colonisation and persistence of *E. faecalis* at this site. However, the Esp_{fs}^{+} strain did not influence histopathological changes in the animal model.[256] The presence of Esp_{fs} also increased cell

hydrophobicity, adherence to abiotic surfaces, and biofilm formation in vitro.[257] Esp_{fs} was suggested to promote colonization of host tissue by direct ligand-binding activity to the extracellular matrix in the human host.[257] This suggestion was based on the fact that Esp_{fs} exhibits characteristics of surface protein receptors designated microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that mediate binding to extracellular matrix proteins.[257]

In addition to a role in adhesion, Esp may also have a function in evasion of the host's immune response (Table 4), based on the observation that the overall structure of both Esp_{fs} and Esp_{fm} are comparable to that of MSCRAMMs.[250,252,255] The Esp_{fs} and Esp_{fm} proteins are similar in sequence and global organization. They contain a signal sequence followed by an N-terminal region and a core region that consists of repeat units.[253,255] Overall, the *esp_{fs}* and *esp_{fm}* genes and the Esp_{fs} and Esp_{fm} proteins share 89% identity.[255] Shankar et al.[253] and Eaton and Gasson[255] showed that different Esp^{+} *Enterococcus* strains may exhibit variations in these repeat units, which may lead to the expression of variant proteins that are identical at the amino and carboxy termini, but differ in the number of repeats.[253,255] Such a phenomenon is thought to be related to evasion of the immune response.[253] The C-terminal regions of the Esp proteins contain a membrane-spanning hydrophobic domain and a cell wall anchor motif involved in anchoring the protein to the bacterial surface.

5. Adhesin to Collagen from *E. faecalis* (Ace) and *E. faecium* (Acm)

Ace and Acm are adhesins (Table 4) that show structural similarity to MSCRAMMs of other gram-positive bacteria, particularly to the collagen-binding protein Cna of *S. aureus*.[258,259] The structural organization of all Ace, Acm, and Cna are similar in that they contain an N-terminal signal sequence followed by the collagen-binding A domain, a B region that consists of repeat units, a cell wall domain with a characteristic LPKTS motif, which is a potential target for sortase, a stretch of hydrophobic residues, which are thought to stretch the membrane followed by a short cytoplasmic charges tail.[259] Because these proteins also contain repeat units and the number of repeats can vary, this protein may also be involved in evasion of the immune response (Table 4) by mechanisms similar to those suggested by Shankar et al.[253] for Esp.

Ace binds not only to collagen (types I and IV) but also to laminin.[260,261] Nallapareddy et al.[261] showed that Ace was expressed by enterococci during human infections. Ninety percent of human sera collected from patients with *E. faecalis* endocarditis reacted with anti-Ace antibodies. Thus, Ace may play an important role in pathogenesis of enterococci, particularly during translocation or when the intestinal epidermal layer is damaged and the underlying extracellular matrix proteins are exposed. As *Enterococcus* cells would become exposed to immune cells at this site, a mechanism for evading the immune system supplied by the same molecule involved in adherence may be an elegant solution for enterococci to increase their chances of survival.

Acm, the collagen-binding protein from *E. faecium*, was shown to bind collagen types I and IV.[259] Nallapareddy et al.[259] showed that particularly the clinical strains of *E. faecium* exhibited binding to collagen type I, while strains from the feces of healthy human volunteers did not bind collagen I. The differences between binding capacity of clinical and community isolate strains were statistically significant, indicating that binding to collagen is a virulence factor. Interestingly, all community *E. faecium* isolates also contained the gene for Acm; however, this gene was in nonfunctional form as a result of

nucleotide deletions or insertion of IS6770-like insertion sequence resulting in frame-shift mutations.

While Ace and Acm share some (47%) amino acid sequence similarity, Acm has a far greater similarity at the primary sequence level (62%) to the collagen-binding protein (Cna) of *S. aureus*.[259] While the similarity of Acm to Ace appears to be confined to the A domain, Acm has similarity to both the A and B domains of Cna.[259]

6. Enterococcus Endocarditis Antigen from *E. faecalis* ($EfaA_{fs}$) or *E. faecium* ($EfaA_{fm}$)

Production of the adhesin-like *E. faecalis* and *E. faecium* endocarditis antigens ($EfaA_{fs}$ and $EfaA_{fm}$, respectively) (Table 4) are considered to be potential virulence determinants, and expression of the EfaA was previously shown to be induced by growth of *E. faecalis* in serum.[262] The $EfaA_{fs}$ antigen shows high homology to adhesins such as FimA, SsaB ScaA, and PsaA from streptococci. $EfaA_{fs}$ was suggested to play a role in adhesion in endocarditis.[262] However, so far only the *efaA*$_{fs}$ gene was shown to influence pathogenicity in animal models.[263] The genetic determinant for production of EfaA has been sequenced and the *efa* operon consists of three genes (*efaC*, *B*, *A*) which have homology to ABC-type metal ion transport systems.[264] The first gene *efaC* encodes an ATP-binding protein, the second (*efaB*) a hydrophobic transmembrane protein, while the third (*efaA*) probably functions as a solute-binding protein receptor for the ABC transporter complex. Low et al.[264] suggested that EfaCBA is a manganese-regulated operon that functions as a high-affinity manganese permease in *E. faecalis*. It plays a role in the infection of human tissues, where the Mn^{2+} availability may be as low as 20 nM.[264]

7. Gelatinase

Gelatinase is an extracellular Zn-metalloprotease (EC 3.4.24.30) that acts on a variety of substrates such as insulin-β chain, collagenous material in tissues, the vasoconstrictor endothelin-1, as well as sex pheromones and their inhibitor peptides.[265] Production of gelatinase increased pathogenicity in an animal model.[263] Kühnen et al.[266] reported that protease-producing *E. faecalis* were common (63.7%) among enterococci isolated from intensive care units in Germany, and Coque et al.[267] showed that 54% of clinical enterococci isolates from patients with endocarditis and other nosocomial infections produced protease. The gene for gelatinase (*gelE*) is located in an operon together with a gene (*sprE*) encoding a serine protease.[268] Mutants containing both defective *gelE* and *sprE* genes led to delayed time to death in a mouse peritonitis model,[263,268] suggesting that both GelE and SprE are important in the infection in this animal model. However, the authors could not determine whether GelE independently influences the outcome of this enterococcal infection.[268]

GelE was shown to cleave fibrin, which was suggested to have important implications in virulence of *E. faecalis* as the secreted protease can damage host tissue and thus allow bacterial migration and spread (Table 4). Waters et al.[265] suggested that enterococci in blood infections and vegetations formed during endocarditis were likely to be coated with polymerized fibrin. Expression of GelE would lead to degradation of this fibrin layer surrounding the bacteria and allow further dissemination of the organism. In addition to its role in virulence, GelE was also shown to affect a variety of important housekeeping functions. For example, GelE clears the bacterial cell surface of misfolded proteins and is also responsible for activation of an autolysin. This muramidase-1 autolysin functions to

reduce chain length.[265] GelE also degrades sex pheromones and their inhibitors. Waters et al.[265] postulated that overall GelE plays a crucial role for dissemination of the organism in high-cell-density environment. Accordingly, degradation of fibrin would aid not only in dissemination, but also in reduction of chain length as a result of autolysin activation. Furthermore, once enterococcal growth reaches high densities, the degradation of sex pheromones decreases aggregation of bacteria, which also increases the potential for dissemination.[265]

The supernatant from a gelatinase-expressing *E. faecalis* strain was also shown to inactivate the antibacterial peptide LL-37.[269] The peptide LL-37 is part of the innate immune system and has been isolated from epithelial cells, neutrophils, and subpopulations of lymphocytes and monocytes. Peptide LL-37 belongs to the family of antimicrobial peptides termed cathelicidins and is activated when cathelicidin hCAP-18 is processed by proteinase 3.[269] Degradation of antimicrobial peptides which are part of the innate immune system thus is a further GelE-associated enterococcal virulence factor (Table 4).

8. Hyaluronidase

Enterococci may produce hyaluronidase, an enzyme that degrades hyaluronic acid, which is a major component of the extracellular matrix (Table 4). Because production of this enzyme was linked to pathogenesis of other microorganisms, it was suggested that it may also play a role in enterococcal pathogenesis. However, there is no direct evidence for the role of hyaluronidase in disease caused by enterococci.[194,270] Recently, the gene sequence for the hyaluronidase gene hyl_{Efm} from an *E. faecium* strain was determined.[270] This gene consisted of 1659 bp, which encodes a putative protein of 533 amino acids with a theoretical molecular weight of 65,051 kDa. The hyaluronidase from *E. faecium* exhibited 42% identity and 60% similarity to a hyaluronidase from *S. pyogenes*.[270] Rice et al.[270] screened a large number of *E. faecium* strains for the incidence of hyl_{Efm} and esp_{fm} genes. These strains were form stool or nonstool origin isolated from both hospitalized and community-based persons. Isolates from animals, waste water, and probiotic strains were also investigated. Rice et al.[270] showed that the presence of esp_{fm} was roughly twice that of hyl_{Efm}, and both genotypes were found primarily in vancomycin-resistant *E. faecium* isolates from nonstool cultures obtained from patients hospitalized in the United States. Their data suggested that specific *E. faecium* strains may be enriched in determinants that make them more likely to cause clinical infections.[270]

9. Capsule

Huebner et al.[271] purified a capsular polysaccharide and determined that it consisted of a repeat structure of kojibiose linked 1,2 to glycerolphosphate. By raising antibodies to this capsular polysaccharide, they used immunogold labeling to show the presence of the capsule surrounding enterococci in electron microscopic studies.[271] They also showed that one third of a sample of 15 clinical *E. faecalis* strains and 7 vancomycin-resistant *E. faecium* strains of clinical origin possessed such capsular polysaccharides.[271] Hancock and Gilmore[272] studied a different capsular polysaccharide from *E. faecalis*, of which the overall composition of the polymer showed some relation to the carbohydrate purified by Huebner et al.[271] However, while the capsular carbohydrate of Huebner et al.[271] contained glucose, glycerol, and phosphate in a 2 : 1 : 2 ratio, that of Hancock and Gilmore[272] contained glucose, galactose, glycerol, and phosphate in a 4 : 1 : 1 : 2 ratio. The nature of the linkages and the structure of the capsular polysaccharide were not determined by

Hancock and Gilmore.[272] Using the capsular polysaccharide-producing strain and an isogenic mutant in a murine cutaneous infection model, Hancock and Gilmore[272] were also able to show that the mutant was more readily cleared from a resulting abscess, as measured by reduction in viable microorganisms from the abdominal lymph nodes that drain this site. This clearly indicated that the production of a capsule does offer some protection to the host's defense mechanisms and that the production of a capsule by some enterococcal strains may play an important role in evasion of the immune response

10. Other Virulence Determinants

Recently, an outbreak of sepsis similar to a toxic shock–like syndrome in humans involving both humans and pigs was attributed to a strain of *E. faecium*.[273] This led the investigators to believe that this particular *Enterococcus* strain may harbor gene(s) encoding toxin(s) similar to streptococcal pyrogenic exotoxins (*spe*).[273] However, toxin structure and the genetic basis for production of this toxin have not yet been studied.

VII. REGULATION OF *ENTEROCOCCUS* VIRULENCE GENE EXPRESSION

Production of aggregation substance (AS) is a tightly regulated phenotype, because autoinduction by a plasmid-bearing donor strain must be prevented. To counteract autoinducing activity, the plasmid-bearing donor cell also excretes a competitive inhibitor, which prevents self-induction and also provides the threshold that allows recipients in the immediate environment to overcome their inhibitory activity with their secreted pheromone.[274] For Asc10, the AS of the sex pheromone plasmid pCF10, the inhibitor iCF10 is secreted at an 80-fold excess to the pheromone cCF10.[274] Induction by cCF10 contained in the cell wall is prevented by cell membrane–associated protein PrgY. Induction occurs if neighboring cells tip the balance in favor of cCF10, which is bound in the cell wall by the pheromone-binding protein PrgZ and consequently imported into the cytoplasm by an Opp (oligopeptide permease) system. The pheromone then interacts with regulatory protein PrgX to allow AS expression. PrgX also controls the transcription of the prgQ promoter, which allows production of iCF10.[274]

Hirt and coworkers[274] showed that the AS of pCF10 is actually induced in vivo and could increase pathogenicity, as measured by size of aortic valve vegetation in a rabbit endocarditis model. In addition, they showed that the expression of AS conferred a survival advantage to cells harboring the plasmid and led to a highly efficient transfer of plasmid. The involvement of the pheromone-sensing system for in AS expression in plasma was confirmed by the absence of AS induction in a mutant lacking the pheromone-sensing protein prgZ. An interaction of plasma components with the inhibitor peptide iCF10 was proposed as affecting the mating behavior.[274]

Production of cytolysin is also a regulated phenotype. Regulation is based on autoinduction and a two-component regulatory system that responds to quorum sensing.[275] The genes necessary for cytolysin production include $cylL_L$, $cylL_S$ (encode structural cytolysin subunits), *cylM* (encodes protein for intracellular modification of cytolysin), *cylB* (encodes ABC transporter protein), *cylA* (encodes protein for extracellular cytolysin activation), and *cylI* (encodes immunity protein), which are arranged in a collinear fashion. Upstream of these biosynthesis and immunity genes on the opposite DNA strand are two ORFs (*cylR2* and *cylR1*), which encode regulatory proteins, consisting of a nonglobular, α-helical

protein with a helix-turn-helix DNA-binding motif (CylR2) and an α-helical protein with three predicted transmembrane domains (CylR1). Together these were shown to repress the cytolysin operon. The inducer for expression of cytolysin was shown to be the smaller, active cytolysin subunit $CylL_{S''}$, and autoinduction was shown to be dependent on cell-density, i.e., by a quorum-sensing mechanism.[275] Unlike other well-known quorum-sensing systems, this two-component regulatory system did not consist of a protein histidine kinase and a response regulator, rather it depends on a small helix-turn-helix DNA-binding protein and a transmembrane protein of unknown function.[275]

The *Enterococcus faecalis* endocarditis antigen $EfaA_{fs}$, as mentioned above, is regulated by Mn^{2+}. The *efaCBA* operon encodes a putative ABC transporter (Efa permease), of which the EfaA component forms the endocarditis antigen. Transcription of the *efaCBA* and EfaA production is repressed by Mn^{2+} by a Mn^{2+}-responsive transcriptional regulator EfaR, which shares 27% identity with the *Corynebacterium diphtheria* diphtheria toxin repressor DtxR.[264] Low et al.[264] suggested that when Mn^{2+} is abundant, intracellular levels rise, resulting in EfaR-Mn^{2+} complexes that bind the *efaCBA* promoter, inhibiting transcription and hence reducing Mn^{2+} uptake. However, if bacteria encounter host tissues or human serum where Mn^{2+} availability is low, the EfaR apoprotein cannot bind the *efaC* promoter, derepressing *efaCBA* expression and hence increasing Efa permease levels and Mn^{2+} scavenging. This may increase the survival of enterococci in the human environment and thus contribute to virulence.

Production of gelatinase is another regulated phenotype. Upstream of the *E. faecalis gelE* and *sprE* genes, there are three genes designated *fsr* (for *E. faecalis* regulator) that regulate the expression of *gelE* and *sprE*. These genes have homology with the *Staphylococcus aureus agr* genes. In *S. aureus*, the *agr/hld* locus contains five genes that encode a quorum-sensing system that regulates the expression of virulence factors.[276–278] The Agr regulatory system upregulates the expression of secreted proteins such as α-toxin, β-toxin, δ-toxin, enterotoxin B, toxic shock syndrome toxin 1, and serine protease and downregulates surface proteins such as protein A, coagulase, and fibronectin-binding protein.[268,276] In this system, *agrA* and *agrC* encode a response regulator and a sensor transducer, respectively, while *agrD* encodes a pheromone peptide that acts as an autoinducer.[268]

Qin et al.[268] demonstrated that for the Fsr system, a cyclic peptide termed gelatinase biosynthesis-activated pheromone (GBAP) is the autoinducer.[279,280] The amino acid sequence of this peptide corresponds to the C-terminal part of a 242-amino-acid protein encoded by *fsrB*.[279] The FsrA protein has 38% similarity to the AgrA protein that encodes the response regulator in the *S. aureus* Agr system, while FsrC has 36% similarity to the ArgC protein that is the sensor transducer of the Agr system.[268] Homology of the Fsr system of *E. faecalis* to the Agr system of *S. aureus*, which plays such an important role in global regulation of *S. aureus* virulence, leads to the question whether the Fsr system plays a similar role in virulence regulation. So far, the Fsr system is only known to regulate two genes in *E. faecalis*, the gelatinase gene, *gelE*, and the serine protease gene, *sprE*. It was demonstrated that an *fsrB* deletion mutant attenuated the virulence in *Caennorhabditis elegans* and a mouse peritonitis model, as well as a rabbit endophthalmitis model, indicating the importance of this gene in virulence.[281,282]

Teng and coworkers[283] studied virulence of enterococci by disrupting two-component regulatory systems in *Enterococcus faecalis*. Such a two-component regulatory system, as mentioned above for gelatinase regulation, consists of a protein histidine kinase and a response regulator protein pair. Using the genome sequence information of *E. faecalis* V583 obtained from The Institute of Genomic Research (TIGR), they

identified 11 homologues to the PhoP-PhoS global two-component regulatory system of *Bacillus subtilis*.[283] Seven of these pairs were disrupted in *E. faecalis* strain OG1RF and one mutant, disrupted in the *etaR* gene of the gene pair designated *etaRS*, showed a delayed killing and a higher lethal dose in a mouse peritonitis model. In addition, they showed that the mutant was more sensitive to low pH and high temperature than the wild-type strain, indicating that *etaRS* may regulate different operon(s) involved in virulence and stress response.[283]

Shepard and Gilmore[284] used real-time PCR to study virulence gene expression and show that AS, Esp, Ace, EfaA, and Gel are induced in serum or urine. However, both environment and growth phase variations were observed, demonstrating the occurrence of uncharacterized control mechanisms for gene expression that may play an important role in vivo.[284]

VIII. INCIDENCE OF VIRULENCE FACTORS AMONG ENTEROCOCCI FROM FOOD

Much progress has been made in determining virulence factors from clinical enterococcal isolates using molecular biological techniques and model animal experiments as described above. Moreover, a number of studies also concerned the incidence of virulence factors of enterococci isolated from foods. Such studies may allow evaluation of the safety of strains intended for use as probiotics or starter cultures.

Eaton and Gasson[254] showed that enterococcal virulence factors were present in food and medical isolates, as well as strains used as starter cultures. However, the incidence of virulence factors was higher among the medical strains than food isolates, and the lowest incidence was observed for starter strains. Strains of *E. faecalis* harbored multiple virulence determinants, while *E. faecium* strains were generally clear of virulence determinants.[254] A similarly low incidence of virulence factors was observed among *E. faecium* strains isolated from food in a previous study, in which only a few strains produced either haemolysin (8.3%) or Esp (2.1%).[282] However, *E. faecalis* strains also harbored multiple virulence determinants, with a much higher incidence than in *E. faecium*. Semedo et al.[286] showed that virulence determinants occurred among food, commensal and clinical isolates of enterococci, but that virulence determinants were significantly associated with a high virulence potential, whereas food and commensal strains harbored fewer virulence determinants. The finding that virulence determinants such as cytolysin, Esp, $EfaA_{fs}$, and $EfaA_{fm}$ were found also in other enterococcal species apart from *E. faecalis* and *E. faecium* led these authors to speculate that the occurrence of virulence determinants is a common trait in the genus *Enterococcus*.[286] The incidence of antibiotic resistance among enterococci from food was also investigated.[55–58,287] These studies showed that although many strains were found resistant to one or more of the antibiotics, the majority of the isolates, especially the *E. faecium*, were sensitive to the clinically relevant antibiotics such as penicillin, ampicillin, streptomycin, and vancomycin.[54,57,285,287] Nevertheless, the occurrence of vancomycin-resistant strains and strains with multiple antibiotic resistances was reported,[287,285] which gives rise to concern.

IX. TRANSMISSION ROUTES AND TRANSFER OF VIRULENCE DETERMINANTS/ANTIBIOTIC RESISTANCES

Evidence is gathering that enterococci from the environment or from foods can survive in and, at least transiently, establish themselves in the human gastrointestinal tract. The study

of Gelsomino et al.[35] mentioned above showed that identical three clones, one of *E. faecalis* and two of *E. casseliflavus*, predominated among farm equipment, milk, cheese, and human fecal samples.[35] In a variety of studies, genetically indistinguishable enterococci have been found in both animals and humans, suggesting that animal-derived enterococci may colonize the human gut.[219,223,225,288,289] Donabedian et al.[290] studied gentamicin-resistant enterococci occurring in food animals, food products from animals of the same species, and feces from humans and found that isolates with indistinguishable PFGE patterns could be recovered from food and from human stool. Their results indicated a dissemination of gentamicin-resistant enterococci from food-producing animals to humans through the food supply.[290] In contrast, Willems et al.[291] investigated vancomycin-resistant *E. faecium* strains from hospitalized patients, nonhospitalized persons, and various animal sources by AFLP genotyping. These investigators suggested that there was a noticeable host specificity. However, in a genotypic study on a large number of *E. faecium* strains from food, veterinary, and human sources isolated from different geographical origins, Vancanneyt et al.[36] could not confirm such a host specificity.

Whether host specific or not, it does appear that enterococci can be naturally transmitted from food animals or foods to the human gastrointestinal tract. One obvious question, then, is how well these bacteria are able to establish themselves in this environment. Studies done so far suggest that they will be present only transiently. Berchieri[292] showed that ingestion of a vancomycin-resistant strain isolated from a chicken resulted in colonization of his own gut for 20 days. Lund et al.[184] showed that a probiotic *E. faecium* strain could not be recovered from the feces of human volunteers 31 days after ceased intake. Sørensen et al.[293] showed that vancomycin- or streptogramin-resistant *E. faecium* strains isolated from food which were given to human volunteers led to only transient intestinal carriage and could not be isolated after 35 days. In all these studies, however, the human volunteers were healthy subjects. It can be speculated that such colonization may not be of a transient nature for debilitated persons and/or persons who receive antibiotic treatment. It has been shown, for example, that enterococci may show a high colonization potential in antibiotic-treated mice.[294,295]

Noting that transient colonization of environmental or food strains can occur raises the question whether there can be transfer of virulence factors or antibiotic resistance genes from strains ingested with food to gastrointestinal strains. Eaton and Gasson[254] studied the probability of gene transfer to starter culture strains in vitro by showing that virulence genes on a pheromone-response plasmid could be transferred to strains of *E. faecalis* used as starter cultures in food. However, they were not able to transfer virulence genes into strains of *E. faecium* starter cultures.[254] Lund and Edlund[296] showed that vancomycin-resistant genes could be transferred to a probiotic *E. faecium* strain in filter mating experiments. The same could be shown for the transfer to probiotic *E. faecium* strains used in animal nutrition by Klein and Pack.[297] However, in the study of Klein and Pack[297] the transfer rate was considerable lower than to a clinical *E. faecium* control strain. The possibility of transfer of virulence factors under in vivo conditions was studied by Huycke,[298] using a hamster model of enterococcal intestinal overgrowth. They showed that pheromone-responsive plasmids carrying either antibiotic or cytolysin genes could be effectively transferred in the hamster gastrointestinal tract, even in the absence of selective pressure with antibiotics.[298] Licht et al.[299] used a new animal model, the streptomycin-treated minipig, to show that the pheromone-response plasmid pCF10 could be transferred in the gastrointestinal tract to other *E. faecalis* strains, again even when there was no selective pressure with antibiotic.[299] From the above

examples it is noticeable that for such gene transfer studies the pheromone-response plasmids were often used, which have a natural high transfer frequency.[229] This may exaggerate the transferability rates of virulence factors or antibiotic resistance genes that are located on other type of plasmids or the transfer of nonpheromone plasmids to other enterococcal strains that generally do not harbor pheromone-response plasmids such as *E. faecium* strains.

X. SAFETY OF ENTEROCOCCI STRAINS FOR USE AS STARTER CULTURES OR AS PROBIOTICS

The incidence of virulence determinants among food isolates studied so far appears to be strain specific.[254,285] One problem associated with risk assessment on the basis of virulence-determinant investigations is that knowledge is limited regarding the type and combinations of virulence factor(s) that are decisive for pathogenic potential. This is especially true in the light of findings that virulence determinants such as aggregation substance, adhesins, and cytolysin appear to be common in the genus *Enterococcus* and are frequently encountered in food strains.[254,285,286] It may be argued that strains that possess multiple virulence determinants associated with various stages of infection (colonization/adherence, translocation, evasion of the immune response, and induction of pathological changes) pose a higher risk than strains that possess a single virulence determinant (e.g., adhesion ability). This may also be deduced from the fact that clinical enterococci strains appear to harbor more virulence factors than food or commensal strains.[254,286] However, clear data about the relative importance of single virulence determinants in food strains are sorely lacking. Furthermore, the question as to whether food strains possess an intrinsically lower pathogenic potential than clinical isolates has still not been fully answered. In a genotyping study of *E. faecium* strains from humans, animals, and food, Vancanneyt et al.[36] found that all human clinical isolates of *E. faecium* fell into a defined subgroup, suggesting that there may be a genetic basis for strains associated with human disease. Thus, virulent subpopulations of strains of a species may exist. Furthermore, there is still not sufficient knowledge on all virulence factors, as well as a lack of detailed comparative studies between clinical and food isolates. It would be interesting to know, for example, whether food strains may also possess capsules and whether *fsr* genes occur in food strains. Furthermore, are there other, so-far unrecognized virulence genes that are regulated by the *fsr* locus or by other regulatory genes such as *etaRS* or *efaR* (see above)? Low et al.[264] found EfaR boxes in the promoter regions of several genes, including two genes that encode natural resistance–associated macrophage protein (NRAMP) homologues. This suggested that EfaR may be a global regulator, which may also be involved in regulation of as yet unidentified virulence determinants. Questions about unrecognized virulence determinants may not be answered immediately, but there is a need for safety decisions to be made. For practical present-day safety investigations, therefore, it can be suggested that if an *Enterococcus* strain is considered for use as starter culture or as a probiotic, each particular strain should be carefully evaluated for the presence of all known virulence factors in order to assess the potential risk for its use. Ideally, such strains should harbor no virulence determinants and should be sensitive to clinically relevant antibiotics. In general, *E. faecium* and other enterococci strains appear to pose a lower risk for use in foods, because strains of these species generally harbor fewer recognized virulence determinants than *E. faecalis*, and the incidence of such virulence determinants appears to be low. The opposite applies to *E. faecalis* strains, and this

may be a result of the presence of pheromone-response plasmids which encode virulence factors.

Because enterococci possess different gene transfer mechanisms (e.g., pheromone-responsive plasmids, conjugative and nonconjugative plasmids, and transposons), it is feared that enterococci may readily acquire these determinants from other enterococcal strains. This represents a possible risk related to the use of enterococci as probiotics or starter cultures. Such a risk would probably be higher for *E. faecalis* strains, as the gene transfer mechanism based on pheromone response plasmids is known to generally occur in this species,[229] although the presence of sex pheromone plasmids in *E. faecium* has been reported.[300] Knowledge on the transfer rates of plasmids to the strain intended for use as starter or probiotic culture would be of great value to assess the likelihood of in vivo transfer. Should such transfer occur, again it would be of value to know the impact of transfer of a single or more virulence determinants to the relative pathogenicity of the recipient strain.

The above discussion shows that the use of enterococci in foods or as probiotic cultures clearly represents a controversial issue in the context of food safety. One should, however, not forget that enterococci are typically associated with the human environment and in particular with the human GI tract. They are also involved in most traditional food fermentations studied thus far, where they appear to play at least some positive role in the development of product-specific typical characteristics. Moreover, particular strains may be considered to be opportunistic pathogens, but it is doubtful that they would cause disease in healthy humans. This is supported by the fact that although food enterococcal strains have been described that harbor single or multiple virulence factors, the morbidity of healthy humans resulting from foodborne enterococcal infections appears to be very low. Furthermore, there are probiotic strains on the market with a long history of safe use and large-scale commercial application. Thus the host factors (i.e., physiological condition, underlying disease, immunosuppression) appear to play a key role in the establishment of an infection with enterococci, and contact of those at risk with these bacteria should be minimized.

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7

Genetics of Lactic Acid Bacteria

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I. INTRODUCTION

The title of this chapter in previous editions of this volume, "Genetic Modification of Lactic Acid Bacteria," aptly reflected the level of knowledge about this subject in the 1990s. The recombinant DNA techniques were developed for lactic acid bacteria (LAB) before a profound knowledge of the genetics of these organisms existed, in contrast to *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae*, and other microorganisms that had been subject to intense biochemical and genetic studies for decades before the advent of genetic engineering.

This situation has changed. By combining recombinant DNA techniques and other recently developed molecular biological methods in the study of LAB, an overview of the actual genetics of these bacteria is starting to emerge. The first nucleotide sequences of the total genomes of LAB are becoming available, and consequently a new, more profound understanding about their physiology and metabolic potential is currently under development. It is to be hoped that this understanding will result not only in more advanced basic and fundamental knowledge, but also in the realization of the expectations regarding the eventual practical applications of genetic techniques to LAB.

The following chapter is an attempt to give both a brief outline of the history of the field and a more detailed account of the latest developments. The mass of information just becoming available makes this a challenging task, and presentation of all the interesting

research within the scope of this chapter is not possible. We hope that the reader will, however, find this chapter useful as a basic introduction to the genetics of LAB.

II. GENOMICS OF LAB

A. Functional Maps of Genomes

The analysis of the chromosomes of strains belonging to genera of the LAB group commenced in the early 1990s and since that time has progressed at an impressive rate. Estimation of the genome size and physical maps were the first results achieved (reviewed in Ref. [1]). The first approach for calculating genome size and intraspecies variability, before genome sequencing, was based on pulsed-field gel electrophoresis (PFGE). This technique is still widely used and has yielded macrorestriction patterns of dozens of strains belonging to almost all the major species of the LAB genera. In some cases only the size of the chromosome was calculated, but physical maps have also been deduced. Recent data obtained by genome sequencing have confirmed the accuracy of the size estimations obtained by means of PFGE. In *Lactococcus lactis* IL1403, PFGE estimated a size of 2.42 Mb,[2] while in the case of *Lactobacillus gasseri* ATCC 33323, the genome size estimated by PFGE was 1.96 Mb.[3] Genome sequences resulted in sizes of 2.36 and 1.84 Mb, respectively.

The amount of data accumulated in a little more than 10 years of PFGE studies has indicated that the majority of LAB have relatively small genome sizes. The only species with a chromosome size greatly exceeding 3 Mb is *Lb. plantarum*,[4] which has been isolated from a number of environments (plants, intestinal and urogenital tracts, dairy products, fermented foods and feeds) and which is not restricted to specific ecological niches such as milk or the intestinal tract. This observation indicates that environmental adaptation of LAB is not only based on gene inactivation, as suggested by Morishita et al. in 1981,[5] but could also involve significant reduction in the size of the genome. However, *Lb. paracasei* strains, which belong to the same subgroup of the genus *Lactobacillus* (the so-called streptobacteria or facultative heterofermentative *Lactobacillus*) and which have been isolated from similar habitats as *Lb. plantarum*, seem to have a smaller genome size: 2.17 Mb as calculated by PFGE analysis[6] and 2.5 Mb according to the ongoing sequencing projects.[7] While it is probably too early to reach general conclusions about this matter, it is clear is that most LAB have small chromosomes.

PFGE analysis has also provided clear evidence of a large strain-to-strain variability in the macrorestriction profiles of LAB, and it is used as powerful strain-typing technique. The percentage of co-migrating fragments in closely related strains of lactococci was calculated to be 80%,[8] in contrast to 20–40% in nonrelated strains. In a similar way the DICE coefficient of similarity calculated for strains of *Lb. helveticus* strains ranged from 26 to 100%.[9]

Strain-dependent variability also includes, in some cases, differences in genome size. In general, whenever closely related strains have been analyzed, differences of 2–3 Kb have been detected.[10] These differences could involve a sufficient number of genes to have an impact on the physiological and the technological properties of strains.

Restriction map–based genetic maps have been obtained for *Lactococcus lactis, Streptococcus thermophilus, Oenococcus oeni, Lb. sakei*, and *Lb. gasseri* by hybridizing gene probes to the mapped restriction fragments.[3,11–14] The first genetic maps were obtained for lactococci and have provided an impressive demonstration of the plasticity of the LAB genome, as they revealed the rearrangements involving large sections of the

chromosome. For example, the maps of two *Lc. lactis* subsp. *cremoris* strains, MG1363 and FG2, have an inversion of approximately 40% of the chromosome when compared with the maps of two *Lc. lactis* subsp. *lactis* strains, DL11 and IL1403.[1,10,15] This inversion has been considered related to the origin of the *cremoris* subspecies. A closer view of the *cremoris* strains provided further evidence of the plasticity of lactococcal chromosomes as four regions of the chromosome of the strain FG2 were found to occupy a different and inverted position with respect to the strain MG1363.[1]

Genomic instability has been detected by means of genetic mapping in clones of the *S. thermophilus* A054 strain spontaneously obtained by subculturing the wild-type strain. Two deletions occurred in each clone, one of which was due to the recombination between two adjacent *rrn* operons.[11,16]

Two strains of *O. oeni* have been genetically mapped.[12,13] Comparative analysis revealed extensive conservation of loci order, even though the two strains were representative, on the basis of macrorestriction profiles and ribotyping, of two divergent clusters of the species.[13]

Genetic mapping of lactobacilli was achieved only recently. In *Lb. sakei* 23K,[14] seven *rrn* loci (coding for ribosomal RNA genes) were found and a total of 47 gene clusters have been mapped, while the chromosome size was estimated to be 1.84 Mb. *Lb. gasseri* ATCC 33323 is another well-studied strain, as it has been mapped[3] and also subject to a sequencing project.[7] Mapping analysis revealed that the chromosome contained six *rrn* operons, one of which was inverted in orientation with respect to the others. This could be an element of instability in the chromosomal structure, as demonstrated in other LAB.[17,18] Each *rrn* operon contained a single copy of each of the three *rrn* genes-23S rRNA (*rrl*), 16S rRNA (*rrs*), and 5S rRNA (*rrf*). PFGE analysis confirmed the presence of a linear plasmid of 48.5 Kb in this strain. The evidence of linear, extra-chromosomal elements in strains of *Lb. gasseri* was first obtained by Roussel et al. in 1993[19] by means of PFGE analysis (see Sec. III. A).

B. Genomic Sequences

As a consequence of the small genome size of LAB and the availability of high-throughput sequencing facilities, microbial genomics is developing fast for this group of bacteria; more than 20 strains (Table 1) are being sequenced,[7] but only a few have been finished and annotated, and it is therefore presently impossible to provide an accurate and updated review. This section therefore provides general considerations on the major advances made in the functional and comparative genomics of food microorganisms, with a special focus on the two LAB genomes that, at the time of writing, have been annotated by the sequencing groups.

1. The *Lactococcus lactis* Genome and Its Relationship to the Metabolic Capacity of the Species

Lc. lactis IL 1403 was the first lactic acid bacterium whose genome was completely sequenced and analyzed.[20] The presence of insertion sequences (IS) (see Sec. IV) is massive: 43 IS for a total of 42 Kb belonging to six different IS groups. This could explain the high degree of genetic plasticity encountered in LAB. The distribution of the IS elements through the 2.36 Mb of the chromosome strongly suggests that this genome may be the result of a recent recombination event between two closely related genomes: IS belonging to the IS981, IS983, and IS1077 families are unevenly distributed, which could be

Table 1 Finished and Ongoing Sequencing Projects: Where to Find Information

Species and strain	Web site(s) or Acc. number
Lc. lactis subsp. *lactis* IL1403	http://wit.integratedgenomics.com/GOLD/ http://www.ebi.ac.uk/proteome/ http://mbgd.genome.ad.jp/ http://pedant.gsf.de/ http://www.ncbi.nlm.nih.gov
Lc. lactis subsp. *cremoris* MG1363	http://wit.integratedgenomics.com/GOLD/ Acc. BH770319-BH771051
Lc. lactis subsp. *cremoris* SK11	http://wit.integratedgenomics.com/GOLD/
Lb. plantarum WCFS1	http://www.cmbi.kun.nl/lactobacillus/
Bifidobacterium longum NCC2705	http://www.ncbi.nlm.nih.gov
Bifidobacterium longum DJO10A	http://www.jgi.doe.gov
Leuconostoc mesenteroides LA81/ ATCC8293	http://www.jgi.doe.gov
Lb. gasseri ATCC 33323	http://www.jgi.doe.gov
Lb. acidophilus NCFM/ ATCC700396	http://www.tigr.org/
Lb. rhamnosus HN001	http://wit.integratedgenomics.com/GOLD
Lb.brevis ATCC367	ftp://ftp.jgi-psf.org/pub/JGI_data/Microbial/ Lactobacillus_brevis/021106/
Bifidobacterium breve NCIMB8807	http://wit.integratedgenomics.com/GOLD
Lb. paracasei ATCC 334	ftp://ftp.jgi-psf.org/pub/JGI_data/Microbial/ Lactobacillus_casei/
Lb. sakei 23K	
Lb. helveticus CNRZ32	http://wit.integratedgenomics.com/GOLD
Lb. casei BL23	
Lb. delbrueckii subsp. *bulgaricus* ATCC BAA-365	ftp://ftp.jgi-psf.org/pub/JGI_data/Microbial/ Lactobacillus_delbrueckii/
Lb. delbrueckii subsp. *bulgaricus* ATCC 11842	http://www.genoscope.fr/
Lb. johnsonii La1 (NCC2761)	http://wit.integratedgenomics.com/GOLD
Streptococcus thermophilus LMG 18311	http://www.tigr.org/
S. thermophilus LMD-9	http://wit.integratedgenomics.com/GOLD
S. thermophilus ATCC BAA-491	ftp://ftp.jgi-psf.org/pub/JGI_data/Microbial/ streptococcus_thermophilus/020930/
S. thermophilus CNRZ 1066	http://wit.integratedgenomics.com/GOLD
O. oeni PSU 1/ATCC BAA331	http://www.jgi.doe.gov
P. pentosaceus ATCC 25745	http://www.jgi.doe.gov

explained by a lateral transfer from a donor *Lactocccus* strain, carrying one type of IS, to a recipient strain carrying another type of IS. Prophages are also present with either complete or defective genomes.

The genome plasticity of *Lc. lactis* strains has been further demonstrated by Le Bourgeois et al.[21] Nine strains of the *Lc. lactis* subsp. *cremoris* (NCDO712, NCDO505,

NCDO2031, NCDO763, MMS36, C2, LM0230, LM2301, and MG1363) were studied by pulsed-field gel electrophoresis. These strains were considered adequate for the investigation of genome plasticity because they were described as belonging to the same genetic lineage. Several macrorestriction fragment length polymorphisms (RFLP) have been found, and Southern hybridization analysis correlated the polymorphic regions with genetic events such as chromosomal inversion, integration of prophage DNA, and location of the transposon-like structures. Comparison with 2236 predicted proteins of *S. pneumoniae*, another gram-positive bacterium with low guanine and cytosine (GC) content, revealed that 905 are highly similar to those from *Lc. lactis* IL1403.

Biosynthetic capabilities of lactococci, as revealed by genome analysis, include genes for the biosynthesis of 20 amino acids. However, it is well known that this bacterium requires at least 6 amino acids to grow in a synthetic medium. The presence of mutation potentially leading to inactivation of the respective gene was detected in 30 of the genetic determinants involved in amino acid biosynthesis.[20]

De novo biosynthesis of purine compounds has been suggested by the presence of 57 genes involved in this metabolism, which is of paramount relevance for a bacterium adapted to ferment milk, a substrate containing low levels of these DNA building blocks.

Surprisingly, genome analysis of this facultatively anaerobic bacterium has led to the discovery that functions required for aerobic respiration are also present among the chromosomal genes; therefore, *Lc. lactis* cannot be considered an exclusively fermentative microorganism.

2. The Genomic Sequence of *Lactobacillus plantarum*

The complete genome sequence of *Lb. plantarum* WCFS1, a derivative of the human isolate NCIMB88226, has been published.[22] It is a 3.3 Mb circular chromosome with 3052 potential protein-encoding genes. It has been possible to assign a biological function to more that 2500 predicted proteins, but 344 hypothetical proteins had no database matches.

The most striking feature resulting from the sequence analysis was the high potential of this bacterium to import and metabolize a range of carbon sources. On the other hand, this strain seems to lack the primary enzyme for protein breakdown, the extracellular protease that has been found in *Lc. lactis.* Quite surprisingly, a gene cluster encoding for nonribosomal peptide synthesis has been found, the first example of such a system in LAB.

3. Comparative Sequence Data Available from Other LAB

A comparative analysis of the genomes of two strains of lactobacilli belonging to species inhabiting the intestinal tract has also been performed.[23] Analysis of *Lb. acidophilus* NCFM and the *Lb. gasseri* ATCC 33323 genomes showed some regions of extended homologies. These regions appear to be fragmented internally, indicating only localized similarities. No long stretches of continuous homology could be detected. Besides the linear regions of similarity, three large-scale genome inversions were detected. One of these inversions causes a strong asymmetrical GC skew distribution. A hypothetical reversion of this region would not only restore the GC skew but also reestablish a direct similarity between the 0.48–0.52 Mb region of *Lb. gasseri* and the 1.96–1.99 Mb region of *Lb. acidophilus.*

This situation strongly resembles what has been previously described in the chromosomes of the two subspecies of *Lc. lactis*,[15] but the DNA level comparison between *Lb. acidophilus* NCFM and *Lc. lactis* IL1403 showed no extended region of simi-

larity.[23] Stress operons in *Lb. acidophilus* NCFM[24] have been shown to have a high degree of similarity with the same genetic structure found in distant *Lactobacillus* species such as *Lb. sakei.*

A preliminary characterization of the genome of *S. thermophilus* CNRZ 1066[25] has shown the presence of a high number of truncated ORFs; this observation could indicate an adaptation of this bacterium to a nutrient-rich environment such as milk. The sequencing of the entire chromosome of *O. oeni* Lo84.13[26] has revealed the presence of 1806 putative coding sequences, distributed in a 1.82 Mb circular genome.

C. Genome Plasticity Versus Environment

The genomes of LAB sequenced and commented on suggest adaptation to the environment, which is strongly reflected in the genome organization. If we take into consideration the *Lc. lactis* genome, it turns out that the genes involved in basic information processes and gene regulation are highly similar to those found in *Bacillus subtilis*, which has a larger genome. However, the narrow range of habitats occupied by *Lc. lactis* (mainly milk) is reflected by the presence in *Lc. lactis* of only three potential sigma factors and eight two-component regulatory systems, in contrast to 18 and 34 such systems, respectively, in *B. subtilis.*

Genome analysis has confirmed the presence of genes involved in oligosaccharide utilization in *Lb. acidophilus* NCFM.[27] The ability to colonize a wide variety of habitats is typical for *Lb. plantarum*, and this is apparently reflected in a number of genes involved in sugar transport and utilization in its genome. *Lb. plantarum* belongs to the so-called facultative heterofermentative (streptobacteria) subgroup of *Lactobacillus*, in which the Embden-Mayerhof pathway is active for six-carbon sugars and the phosphoketolase pathway for pentoses. Quorum sensing genes have been found in *Lb. plantarum* WCFS1,[28] but it is unclear if these genes have a real role in this intestinal isolate.

At present we cannot make general or final conclusions about the initial data provided by comparative genomics. As noted earlier, *Lb. plantarum* has a chromosomal size approximately 1 Mb larger than the genome of *Lb. paracasei.* Both species have been isolated from the same types of environments. LAB form a very large and diverse group of bacteria, which could have adopted different strategies for surviving in the same habitats. The ongoing sequencing projects (Table 1) will provide evidence for or against this hypothesis.

Although the genetics of *Bifidobacterium* is not specifically addressed in this chapter, it is worthwhile to mention results achieved by means of bioinformatic analysis of the 2.26 Mb genome sequence of an infant-derived strain of *Bifidobacterium longum.*[29] Among the 1730 possible coding sequences identified, several physiological traits could, at least partially, explain the successful adaptation of this bacterium to one peculiar ecological niche, the colon. A large number of the predicted proteins appear to be involved in the catabolism of oligosaccharides. This ability to scavenge a variety of nutrients could explain the observed competitiveness and persistence of bifidobacteria in the colon. However, in this case we have to be prudent and wait for more data before concluding a tight linkage between catabolic potential and gut colonization ability.

D. Proteomics

Proteomics was originally based on two-dimensional electrophoresis of proteins. The technique has been refined in many ways and is now being applied to LAB.[30] While

genomic analysis provides a static view of an organism, proteomics allows a dynamic observation of cellular events of interest in food production. *Lc. lactis* MG1363 and *S. thermophilus* were investigated by means of proteomics, and a reference protein map has been obtained for these bacteria.[31,32] *Lc. lactis* NCDO 763 has been investigated by two-dimensional gel electrophoresis,[33] and 15 proteins were initially identified.

A proteomic approach has been used to investigate the stress response of LAB[31,34] and to analyze differential expression patterns of lactococci under standard conditions and during purine nucleotide starvation.[35] Comparison at the proteome level of two strains of *Lc. lactis* showed an important protein polymorphism. Comparison of the proteomes of glucose- and lactose-grown cells revealed an unexpected link between the nature of the carbon source and the metabolism of pyrimidine nucleotides.[36]

III. PLASMID BIOLOGY OF LAB

The observations made by L. L. McKay at the University of Minnesota about the spontaneous and acriflavine-induced loss of lactose fermentation ability of lactococcal strains suggested involvement of plasmids in this phenotype.[37] Subsequent demonstration of extrachromosomal DNA in the lactococci soon led to the identification of several metabolic plasmids and their functions.[38] Because of their technological importance study of the metabolic plasmids dominated the early stages of genetic studies on LAB (see also Sec. III.B). In general, presence of plasmids is characteristic of many species and genera of LAB, and their study has revealed many aspects that have, in addition to their significance for LAB, a wider relevance in general biology. These include mechanisms to control the plasmid copy number and to prevent the presence in the same cell of two plasmid species sharing too identical replication functions (incompatibility). These mechanisms are intimately linked with the plasmid replication functions.

A. Physical Structure, Replication Mechanisms, Host Range, and Incompatibility

1. Circular Plasmids

The majority of LAB-associated plasmids belong to the standard type of covalently closed circular, autonomously replicating DNA molecules. These plasmids replicate by two basic mechanisms: the rolling circle type of replication or theta replication. Because plasmid host range, stability, and incompatibility are apparently linked to the replication mechanism, a closer look at them is justified.

The Rolling Circle Mechanism. The basic features of this type of replication were reviewed by Gruss and Ehrlich in 1989,[39] and the detailed molecular events by Novick in 1998.[40] Briefly, in this type of replication a nick is first formed by the replication initiation protein (IP) in the so-called plus origin of replication, which is situated in the immediate vicinity of the IP gene. After the nick, a replisome, consisting of a single strand–binding protein, DNA-polymerase III holoenzyme, and a helicase, starts the synthesis of a new strand while displacing the plus strand (Fig. 1). When a cycle has been completed, a double-stranded plasmid and a circular single-stranded intermediate are formed by cutting and religating the replaced minus strand. A new round of DNA

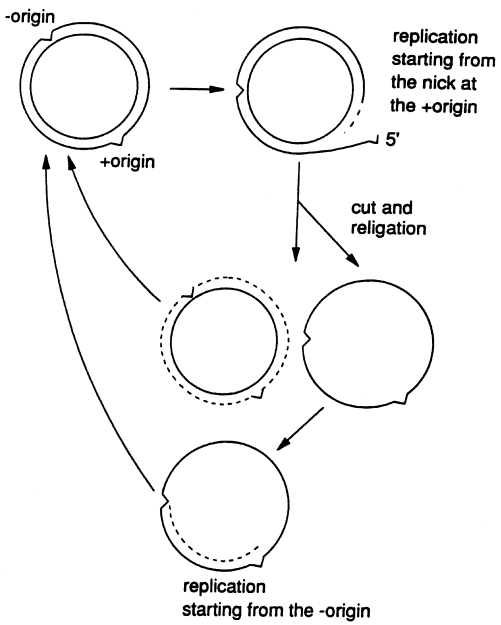

Figure 1 The rolling circle type of plasmid replication. Solid line represents parental DNA and dashed line the newly synthesized strand.

synthesis starts from the minus origin of this intermediate, leading to the formation of another double-stranded plasmid.

The staphylococcal plasmid pT181 has been used as a model to characterize the copy number control and incompatibility functions of rolling circle replicating plasmids. In this plasmid the synthesis of IP is controlled by an attenuation mechanism involving a leader sequence in the IP messenger RNA capable of interacting with antisense RNA coded by a specific replication region site (*cop*). In the presence of antisense RNA, the leader sequence forms a transcription stop signal structure leading to the cessation of transcription and IP synthesis.[41]

The small lactococcal plasmids pVW01 and pSH71, which have been used in cloning vector construction (see Sec. VI. A), are typical examples of plasmids replicating by a rolling circle mechanism. Their plus origins closely resemble that of a small staphylococcal plasmid pE194.[39,42] These plasmids have a broad host range replicating in several other gram-positive hosts and in *E. coli*.[43]

Although these small lactococcal plasmids have a similar mode of replication and share homologies in the replication region with the small staphylococcal plasmids such as pE194 and pC194, the latter usually do not replicate in lactococcal hosts. However, a mutation in a single base pair in the replication region expands the host range of pC194, a chloramphenicol resistance plasmid, into the lactococci.[44] This mutation apparently enhances the promoter controlling the transcription of the IP gene (J.C. Alonso, personal communication).

The rolling circle type of replication seems, in general, to be typical for small plasmids of LAB, several of which have been characterized and totally or partially sequenced. Examples include lactococcal plasmids such as pFX2,[45] the *O. oeni* plasmid

pLo13,[46] the *Lc. lactis* plasmid pCI411,[47] the *Lb. plantarum* plasmid pC30il,[48] *Lb. fermentum* plasmid pLEM3,[49] and pER371 from *S. thermophilus*.[50] The stability of plasmid pGT232 from *Lb. reuteri* has been shown to depend on the presence of the functional minus origin.[51]

Theta-Replicating Plasmids. Theta replication is based on a progressive replication fork with simultaneous synthesis of both new strands. The synthesis can be uni- or bidirectional. No extended single-stranded regions are formed.

The theta-replicating plasmids in LAB are generally of medium or large size, spanning from a few thousand to tens of thousands of bp. These plasmids include large metabolic plasmids (see Sec. III. B), such as lactococcal lactose fermentation and proteinase plasmids,[52,53] citrate permease plasmids,[54] and phage resistance plasmids.[55,56] Other examples are cryptic plasmids, such as pVS40,[57,58] pCI305,[59] and pWV02.[60] The last has an exceptionally small size (3.8 kpb) for a theta-replicating plasmid. The host range of these theta-replicating plasmids is rather limited in comparison to rolling circle plasmids.

The sequences of the lactococcal plasmids mentioned above share a remarkable degree of homology in their respective replication regions. The replication protein gene (*repB*) is preceded by an AT-rich origin of replication (*repA*). One striking feature is the presence of three successive complete and one incomplete 22 bp direct repeats with a general consensus sequence of TATANNNNN(A/T)NAAAAA(A/T)C(T/G)(G/A)TC immediately before the promoter of the *repB* gene. Two inverted repeats, one between the −10 region and the start of *repB*, are also regularly found, as well as two AT-rich short (9–10 bp) repeats further upstream of the 22 bp sequences.

The lactococcal family of theta-replicating plasmids seems also to be generally compatible with each other. After screening of 12 theta-replicating plasmids, two incompatible pairs, pFV1001 and pFV1201 and pJW565 and pFW094, were found.[61] The incompatibility region could be tentatively located within the above-mentioned region of 22 bp direct repeats and the first inverted repeat.

Enterococcal theta-replicating plasmids of pAMβ1 family are other well known examples of theta-replicating plasmids (reviewed in Ref. [62]). They are large (20–60 kpb) conjugative erythromycin-resistance plasmids with a broad host range. Their origin of replication does not have structures resembling the lactococcal *repA* region. Their wide host range and ability to mobilize nonconjugative plasmids (see Sec. V.A) has made them useful in genetic studies of LAB.

Theta-replicating plasmids have been characterized in other species of LAB. It appears that *Lb. plantarum, Enterococcus faecalis, S. bovis, Lb. acidophilus*, and *Tetragenococcus halophilus* represent a group of theta-replicating plasmids sharing a high degree of homology, especially in their *repB* gene[63–65] A small 2.665 bp cryptic plasmid pTXL1 from *Leuconostoc mesenteroides* has been assumed to replicate via the theta-replication mechanism, because typical genetic elements of the rolling circle type of replication were absent and no single-stranded plasmid DNA could be detected.[66]

2. Linear Plasmids in *Lactobacillus*

In addition to circular covalently closed plasmids, the presence of linear plasmid has been suggested in strains of *Lb. gasseri.* Evidence obtained by means of PFGE analysis[3,19] showed that some bands obtained by running PFGE without any restriction digestion were able to migrate at the same relative position even when different switch times

were used. This is an indication of the linear nature of the migrating DNA. Linear plasmids have been discovered in several bacteria, but *Lb. gasseri* seems so far to be the only lactic acid bacterium in which this kind of extra-chromosomal DNA has been detected.

B. Metabolic Plasmids

1. Carbohydrate Fermentation and Proteinase Plasmids

Plasmids coding for important technological properties have naturally attracted much attention since their discovery in the lactococci. In these bacteria the lactose fermentation and proteinase activities are almost invariably associated with relatively large (from 17 to >50 kbp) plasmids.[68] The lactococcal lactose fermentation is based on the phosphoenolpyruvate (PEP)–dependent phosphotransferase system. Lactose enters the cell as lactose-6-phosphate, which is subsequently split into galactose-6-phosphate and glucose by phospho-β-galactosidase. Glucose is further metabolized by the Embden-Meyerhof-Parnas pathway, whereas galactose-6-phosphate is first converted into tagatose diphosphate before it can be split into two triose phosphates and enter the normal glycolytic pathway (for review, see Ref. [68]). The lactococcal proteinases are cell wall–associated enzymes that are very closely related to each other. They form an essential part of an intricate machinery producing and transporting peptides necessary for the growth of the cell (reviewed in Ref. [69]). Except for the lactococci, plasmid linkage of proteinase activity has been reported in at least one strain of *Lb. helveticus*.[70]

The presence of carbohydrate-fermentation plasmids is not as common in lactobacilli as in lactococci. However, examples of lactose fermentation plasmids are known also among lactobacilli. Plasmid linked PEP-dependent lactose phosphotransferase system has been associated with *Lb. casei* strains,[71] and one of the key enzymes of the system, 6-phospho-β-galactosidase, is located on a *Lb. casei* plasmid pLY101.[72] Two different plasmids, one coding for 6-phospho-β-galactosidase and the other for the genes of the tagatose diphosphate pathway, have been characterized in *Lb. acidophilus* TK8912.[73]

In some lactobacilli the plasmid-encoded lactose utilization is based on another mechanism, the lactose permease pathway. Lactose enters the cell unphosphorylated, and is subsequently split by β-galactosidase, the key enzyme of the system. Plasmids coding for β-galactosidase seem to be typical for *Lb. plantarum* strains.[74] It also appears that in many *Lb. plantarum* strains there simultaneously exists both a plasmid-associated and chromosomally coded copy of the gene, possibly reflecting adaptation of these strains to the dairy environment.[75]

The plasmid encoded β-galactosidase system is also common in strains of *Leuconostoc*.[76] In *Le. lactis* both lactose permease and β-galactosidase genes reside on the same plasmid, but do not form an operon. The β-galactosidase gene is coded by two partially overlapping genes (*lacL* and *LacM*), while the permease gene is separated by a 2 kbp DNA fragment containing an IS sequence.[77,78]

As an example of a carbohydrate other than lactose, the utilization of raffinose, a trisaccharide, is linked to relatively large plasmids (36.2–47.3 kbp) in at least three strains of *Pediococcus pentosaceus*.[79]

2. Plasmids Associated with Aroma Production

Diacetyl is an important aroma compound ("butter flavor") in dairy products. In dairy cultures diacetyl is produced from citrate by strains of *Lc. lactis* subsp. *lactis* biovar.

diacetylactis and members of the genus *Leuconostoc.* The key enzyme in the pathway is citrate permease, which mediates the citrate uptake by the cells.

The linkage of citrate permease gene in *Lc. lactis* to small (approximately 8.7 kbp) plasmids was detected relatively early.[80,81] Subsequently, it was found that at least some leuconostocs have a similar system.[82] Although the genes involved in the citrate metabolism share a high degree of homology between the two species, they are differently regulated, in lactococci by external pH and in leuconostocs by citrate.[83,84]

3. Mucoidness

Mucoidness, or the ability to produce extracellular polysaccharides, is a property of some lactococcal strains that have traditionally been used to give body and texture to certain types of Scandinavian fermented milks.[85,86] Several lactococcal plasmids ranging in size between 27 and 47 kbp associated with mucoid phenotype have been identified.[87–89] Genetic analysis of one of the plasmids, pNZ4000, has indicated the involvement of at least 14 genes in the exopolysaccharide production.[90] The complete sequence of this 42.2 kbp plasmid has been elucidated.[91]

Mucoid variants are common among other species of LAB. In at least some strains of *Lb. casei*, mucoidness has been correlated with the presence of plasmids ranging in size from approximately 7 to 30 kbp.[92,93]

C. Plasmids Associated with Bacteriocin Production and Immunity

Bacteriocins are proteins or peptides secreted by bacteria that inhibit the growth of other bacterial strains or species. LAB produce a wide variety of bacteriocins active against other gram-positive bacteria (other species and strains of LAB, *Clostridium, Listeria*, etc.). The bacteriocins are classified into three groups:[94] lantibiotics or bacteriocins containing modified amino acids, such as lanthionine and β-methyllanthionine (class I—bacteriocins), small heat-stable nonlantibiotic peptides (class II), and large heat-labile bacteriocins (class III). Nisin is a well-known example of class I bacteriocins. Although early evidence suggested plasmid involvement in nisin production, it is now well established that the trait is coded by a chromosomally located conjugative transposon (see Sec. V).

Regardless of class, the bacteriocin gene clusters have certain organizational similarities. Typically the bacteriocin gene contains a leader sequence with a double function. The resulting N-terminal leader sequence inhibits the intracellular activity of the bacteriocin while simultaneously providing the signal to the transport system. The immunity gene is usually intimately linked to the bacteriocin gene and expressed concomitantly with it.[94]

1. Plasmid-Encoded Lactococcal Bacteriocins

Historically, one the very first plasmid-associated lactococcal bacteriocins was diplococcin, the production of which is coded by an 81 kbp conjugative plasmid.[95] Apparently no further genetic or molecular biological analysis has been done on this system.

Lacticins represent plasmid-encoded class I bacteriocins. Lacticin 481, coded by a six-gene operon located on a 70 kbp plasmid in *Lc. lactis* ADRIA 85LO30,[96] is a typical example. It is particularly active against *Clostridium tyrobutyricum*.[97] Another plasmid-encoded bacteriocin belonging to the class I bacteriocins is the two-component lantibiotic lacticin 3157 encoded by a 60 kb plasmid, which has been completely sequenced.[98]

Lactococcins belonging to the class II bacteriocins are mainly active against other lactococci. An illustrative example of a plasmid coding for multiple bacteriocins is p9B4-6 from *Lc. lactis* subsp. *cremoris* 9B4.[99] In this plasmid one bacteriocin locus consisting of two genes jointly produced the so-called lactococcin M (low antagonistic activity), while the other region coded for two independent highly active lactococcins A and B.[100,101]

2. Bacteriocin Plasmids from the Lactobacilli

A wide range of both chromosomally encoded and plasmid-linked lactobacillar bacteriocins is known. An example of a lactobacillar lantibiotic is lactocin S, coded by two operons in a 50 kbp plasmid pCIM1 in *Lb. sakei* L45.[94]

Lactacin F is a plasmid-associated two-peptide class II bacteriocin from *Lb. johnsonii.* The genes have been cloned and expressed in *Carnobacterium pisciciola* LV17. The latter strain produces three class II bacteriocins: carnobacteriocins A, B2, and BM1. Carnobacteriocins A and B2 are coded by two separate plasmids, pCP49 and pCP40, while the gene for BM1 is chromosomal. The resulting recombinant *Carnobacterium* strain is capable of producing both lactacin F and carnobacteriocins.[102]

Other examples of plasmid involvement in bacteriocin production are acidocin 8912[103] and acidocin B,[104] both produced by 14 kbp plasmids (pLA103 and pCV461, respectively) in *Lb. acidophilus*, and sakacin A, associated with a 60 kbp plasmid of *Lb. sakei* Lb706.[105]

3. Other Plasmid-Associated Bacteriocins from LAB

Pediocins and pediocin-like bacteriocins are often referred to as class IIa bacteriocins. Because of their strong activity against *Listeria*, they are particularly interesting, for the food industry. They are often plasmid-encoded bacteriocins produced by, among others, *P. acidilactici* as well as by a number of other LAB.[106,107] Mesentericin Y105 is an example of class IIa bacteriocins produced by *Le. mesenteroides* Y105 harboring a 35 kbp production plasmid.[108] Enterocin ON-157 is a recently characterized enterococcal bacteriocin active mainly against other enterococci. Loss of bacteriocin activity is connected with novobiocin-induced curing of a 49 kbp plasmid of the *E. faecium* production strain.[109]

D. Plasmids and Phage Defense Mechanisms

Plasmids associated with increased resistance against bacteriophages are common, especially among the lactococci. Three basic phage resistance mechanisms with different subdivisions are known: inhibition of phage adsorption, restriction/modification (R/M) systems, and abortive infection (Abi) or intracellular inhibition of phage development. Tens of plasmids coding for these mechanisms are known[110,111] (see also Chapter 8). Even a cursory attempt to describe them in this context is not possible here. In the following paragraphs only the best characterized plasmids associated with multiple phage defense mechanisms are presented.

1. Bacteriophage Defence Mechanisms Associated with the Plasmid pNP40

A 65 kbp conjugative plasmid pNP40 from *Lc. lactis* ssp. *lactis* biovar. *diactylactis* DRC3 confers resistance against the bacteriocin nisin and also protects the strain from the attack of a lytic bacteriophage c2.[112] Subsequently, the plasmid has been shown to contain

genes for at least two Abi systems (AbiE and AbiF) and for a mechanism blocking phage DNA injection.[113,114] The fact that this plasmid also codes for cadmium resistance, which can be used as a selective marker, further emphasizes its potential usefulness in engineering dairy starter strains for enhanced phage resistance.[115] Among the other genes characterized in this plasmids are a homolog of *recA* (a gene central in DNA recombination and repair) as well as a gene-sharing homology with *umuC* (a gene involved in the so-called SOS response to DNA damage).[116] These findings suggest a possible role for genes active in DNA recombination and repair in some of the Abi mechanisms.

2. Restriction Modification and Abortive Infection Coded by the Plasmid pTR2030

Lc. lactis ssp. *lactis* ME2 is an exceptionally phage-resistant strain harboring a 46 kbp plasmid pTR2030[117] encoding both an R/M system and an Abi mechanism. The R/M system associated with pT2030, *LlaI*, has been both subcloned and analyzed at the molecular level,[118,119] as well as transferred to heterologous hosts.[120]

E. Antibiotic Resistance Plasmids

1. Lactococcal Antibiotic Resistance Plasmids

Antibiotic resistance plasmids are relatively rare among lactococci. However, a 30 kbp completely sequenced theta-replicating plasmid pK214 coding resistance for streptomycin, tetracycline, and chloramphenicol resistance determinants apparently related to corresponding genes in *Staphylococcus, Listeria*, and *Enterococcus* has been detected in a lactococcal strain isolated from soft cheese. The plasmid also contains five IS elements, three of which apparently originate from *E. faecium*.[121]

2. Antibiotic Resistance Plasmids in Lactobacilli

Early work by Vescovo et al.[122] demonstrated by plasmid curing experiments that in 20 *Lb. acidophilus* strains originating from pig and calf feces several resistances (penicillin, ampicillin, cloxacillin, aminoglycosides, tetracycline, erythromycin, chloramphenicol, bacitracin) were probably plasmid-associated. Plasmid linkage to erythromycin and chloramphenicol resistance has been detected in *Lb. reuteri, Lb. plantarum*, and *Lb. fermentum* strains isolated from poultry, pigs, and pork.[121] An *ermT* carrying 4.2 kbp plasmid p121BS from an unidentified *Lactobacillus* sp. of swine origin conferring resistance to tylosin and erythromycin has been completely sequenced.[123] Plasmid pMD5057 (10.9 kbp) from *Lb. plantarum* of meat origin represents a completely sequenced lactobacillar tetracycline resistance (*tetM*) plasmid.[63]

3. Enterococcal Antibiotic Resistance Plasmids

Resistance to a wide range of antibiotics is typical for enterococci isolated from clinical, food, and fecal sources. In many cases the resistances are located in mobile genetic elements (see Sec. IV), but resistance plasmids, many of which are conjugative, are typical to this genus. Plasmids pAMβ1[124] and pIP501,[125] the former a 26.5 kbp erythromycin resistance plasmid and the latter a 30 kbp chloramphenicol-erythromycin double resistance plasmid, are classic examples of conjugative enterococcal plasmids with a wide host range. These plasmids can be regularly conjugated to other species and genera of LAB (see Refs. [126,127]). Conjugative plasmids carrying genes for erythromycin resistance

(*ermAM*) or tetracycline resistance (*tetM*) have been detected in enterococci isolated from cheese or meat products.[121,128]

F. Other Plasmid-Associated Phenotypes

While plasmids coding for important functional properties have, understandably, attracted much attention and antibiotic resistance plasmids are of interest regarding the safety aspects of LAB, there probably are other plasmid-associated phenotypes worth studying, although more difficult to detect. Certain defense or adaptation mechanisms in some LAB strains are plasmid-encoded. UV resistance and adaptation to heat stress are currently known examples.

1. Ultraviolet Resistance

In *Lc. lactis* ssp. *lactis* IL594, the resistance to ultraviolet (UV) irradiation is plasmid-encoded. The relevant plasmid, pIL7, has a size of 33 kbp, and by cloning experiments the UV-resistance determinant has been located in a 5.4 kbp fragment.[129]

2. Heat Shock Proteins

In some strains of *S. thermophilus* a heat shock protein (Hsp16.4), which may participate in protecting the cell from heat stress, is plasmid encoded.[130,131] In hybridization experiments the gene coding for this heat stress protein gene could also be located on a 7.5 kbp plasmid in one strain of *Lc. lactis* ssp. *cremoris* (out of 24 lactococcal strains screened). The size range of *S. thermophilus* plasmids encoding Hsp16.4 is 2.8–11.0 kbp.[131]

IV. INSERTION SEQUENCES, TRANSPOSONS, AND INTRONS

Different transposable genetic elements are important mechanisms to enhance the genetic mobility and elasticity of bacteria. While the general genetic recombination in LAB as well in other bacteria requires homologous sequences between the participating DNA molecules, the transposable genetic elements require only short (3–10 bp) target sequences in the recipient DNA. While the insertions can occur either at a specific site or apparently randomly, depending on the mobile element, the transpositions introduce mutations and genetic rearrangements. Insertion sequences represent the simplest form of transposable genetic elements. They have a size range of 750–2000 bp and typically contain only the genes necessary for the transposition flanked by short inverted repeats. Numerous IS elements have been characterized, and their characteristics and occurrence in various bacteria groups, including lactococci, lactobacilli, enterococci, leuconostocs, and pediococci, have been extensively reviewed.[132] In contrast to simple IS elements, typical transposons contain additional genes, such as antibiotic resistance determinants or genes involved in conjugative gene transfer (see Sec. V.A), flanked by IS sequences.

Type II introns represent another type of bacterial integrative genetic elements. Introns are sequences interrupting functional genes. They are transcribed to RNA along with the rest of the gene, but subsequently spliced during the processing of mRNA. While introns are common in eukaryotes, their presence in bacteria was a relatively recent finding. So far, among LAB, type II introns have been detected in lactococci.[133]

A. Insertion Sequences

The first IS element in LAB, ISL*1*, was identified in *Lb. casei* by Shimizu-Kadota et al.[134] Subsequently insertion elements have been found both in other species of lactobacilli as well as in lactococci. The two completely sequenced LAB genomes, that of *Lc. lactis* ssp. *lactis* IL1403[20] and *Lb. plantarum* WCFS1,[22] are illustrative examples of the frequency and occurrence of IS elements in the genome.

1. IS Elements in *Lactococcous lactis* ssp. *lactis*

In the *Lc. lactis* ssp. *lactis* IL 1403 genome, altogether 43 copies of IS elements could be found representing six known types (IS*981*, IS*982*, IS*983*, IS*904*, IS*905*, IS*1077*). The copy numbers of individual IS elements range from one (IS*982*, IS*905*) to 15 (IS*983*). IS*981*, IS*904*, and IS*1077* belong to a larger IS*3* family of insertion elements, while many of the IS elements characterized specially in lactococcal plasmids belong to the ISS*1* class of IS*6* family.[132] Formation of cointegrates where the donor and target replicons are separated by two directly repeated IS copies is typical of this family. ISS*1* elements have been shown to cause spontaneous formation of cointegrates between lactococcal plasmids,[135] and they have been successfully used for insertional mutagenization of lactococcal chromosomal genes.[136]

2. IS elements in *Lb. plantarum*

Two classes of transposase-coding regions resembling IS elements, designated as ISP*1* (eight complete copies) and ISP*2* (four complete copies), have been detected in the genome of *Lb. plantarum* WCFS1.[22] ISP*1* represents a typical IS sequence having a homology with IS*1165* of ISL*3* family from *Le. mesenteroides*. The ISP*2* apparently does not have the terminal inverted repeats, and its role or type of eventual transposition is therefore unclear.

B. Transposons

1. Lactococcal Conjugative Transposons Associated with Nisin Synthesis and Sucrose Fermentation

The genes for nisin biosynthesis and immunity as well as for sucrose utilization are known to reside on a 70 kbp conjugative transposon. This block is flanked by direct repeats of TTTTTG, most likely representing a duplication of the target sequence due to transposition. However, no inverted repeats flanking the nisin-sucrose gene block have been identified.[137] The nisin-sucrose transposons vary slightly from strain to strain, which has resulted in different designations (Tn*5301*, Tn*5276*, Tn*5307*, etc.). The conjugative nature of the nisin-sucrose transposons allows for their introduction even to heterologuous hosts, such as dairy enterococci.[138]

Recently transposons associated with sucrose fermentation only have been detected in *Lc. lactis* strains of plant origin.[139] These could be conjugated to sucrose-negative recipients, and the transconjugants contained insertions of variable sizes (50–110 kb) in their chromosome.

2. The ICE*St1* Element of *Streptococcus thermophilus*

In *S. thermophilus* a novel, conjugative, transposable genetic element has been recently characterized.[140] The 34.7 kb element ICE*St1* integrates site-specifically to the gene

coding for putative fructose-1,6-diphosphate aldolase. The proteins associated with the transfer functions of this element shared homology with corresponding proteins of an enterococcal transposon Tn*916* (see Sec. IV.B3). Further comparison of the sequence of ICE*St1* with other elements present in various gram-positive bacteria revealed a common modular structure suggesting a family of conjugative transposons with a circular intermediate involved in the integration process. The term "integrative and conjugative elements" has been suggested for these types of transposons.[141]

3. Enterococcal Transposons

Enterococcal transposon Tn*916*, which confers resistance to tetracycline, was the first conjugative transposon detected.[142] Although several others have been subsequently characterized (Tn*917* and Tn*916*-related Tn*918*, Tn*920*, Tn*925*), (see also Sec. VI.A), Tn*916* is still both the best known and most widely used representative of enterococcal conjugative transposons. This 18 kbp element has subsequently been totally sequenced and analyzed at the molecular level.[143] Characteristic of Tn*916* is a very wide host range spanning both gram-positive and gram-negative bacteria. This property has been utilized in genetic studies of LAB for the insertional inactivation of genes in recipient strains. In the absence of tetracycline, Tn*916* is accurately excised, restoring the original gene structure and function.[144]

From the public health point of view, enterococcal transposons can be problematic. Particularly the transposons associated with vancomycin resistance have received attention, since vancomycin-resistant enterococcal strains can cause persistent nosocomial infections, and there is also the danger of the resistance spreading to other genera, such as pathogenic staphylococci.[145] The best characterized vancomycin resistance transposon Tn*1546* is itself nonconjugative, but it can be effectively mobilized by conjugative elements residing in the same host strain.[146,147]

C. Group II Introns

Characteristic of the group II introns is the formation of a closed circular structure ("lariat") during the splicing event, when the intron RNA is excised from the mRNA. They were first found in the organelle genomes of lower eukaryotes and plants but have subsequently been found in several species of Eubacteria but not in Archeae.[133] Lactococci were the first bacteria in which group II introns were found, and they are regularly associated with conjugative elements or sex factors.[148]

Group II introns have a conserved secondary structure consisting of six domains, three of which (I, V, and VI) have a role in splicing. Domain I contains the exon-binding sites (EBS1, EBS2, and δ). Domain VI contains a protruding adenosine, which is involved in the transesterification reactions essential to the splicing.[149]

Retrohoming is a feature of group II introns. The reverse transcriptase coded by the intron has there a key function. This enzyme, together with some other intron-coded proteins, remains associated with the lariat-like RNA structure after splicing, forming a DNA endonuclease that cuts the target DNA at a site recognized by the exon-binding sites of the intron. The spliced RNA is covalently joined to the DNA, bridging the gap in one strand, while a complementary strand is being synthesized by reverse transcriptase.[133]

The lactococcal intron Ll.ltrB is one of the best characterized bacterial group II introns. Because the specificity of retrohoming depends on the sequences of exon-binding sites, the intron can be retargeted by modifying these sequences. A recent example of the

application of this technique is the targeted insertion of Ll.ltrB into genes coding for malate decarboxylase and tetracycline resistance in the *Lc. lactis* genome.[150]

V. GENE TRANSFER SYSTEMS

Gene transfer between strains, species, and even genera of LAB can occur in vivo with the known natural mechanisms, physiological transformation, transduction, and conjugation. Although the significance of these mechanisms varies greatly among the different species, they contribute to the horizontal gene transfer in the natural habitats of LAB and they can be utilized in the genetic modification of LAB strains. However, for the actual recombinant DNA techniques, reliable in vitro gene transfer mechanisms are essential and have been adapted to the most important LAB.

A. Gene Transfer In Vivo

1. Physiological Transformation

Natural transformation, the first gene transfer mechanism described, was described by F. Griffith in LAB in 1929 in *S. pneumoniae*.[151] Later DNA was shown to be the transforming principle (152; see also Ref. [151]). Transformation by natural competence has been thoroughly studied in *Bacillus subtilis* (for a review, see Ref. [153]), indicating that a number of competence genes need to be expressed for natural competence to take place.

In dairy microorganisms, Møller-Madsen and Jensen[154] first described a natural transformation system in a few strains of *Lc. lactis* by transferring between lactococci the ability to produce a malty flavor. This observation of natural competence has not been satisfactorily confirmed. Møller-Madsen later claimed that only a few strains of lactococci were able to transform by natural competence and that, unfortunately, these strains were lost (A. Møller-Madsen, personal communication). However, a few results indicate that the original observations were not artefacts. Knite (155; see also Ref. [156]) reported the transfer of mannitol and streptomycin resistance in lactococci by natural transformation. Sanders and Nicholson[157] later showed that nonprotoplasted lactococci was able to take up plasmids as well as phage DNA if polyethylene glycol (PEG) was present. Finally, and most convincing, is the identification of the complete competence operons in the *Lc. lactis* IL1403 genome sequence.[158] These observations indicate that lactococcal natural competance has to be reconsidered as a mode of gene transfer in lactococci.

2. Transduction

Transduction is the transfer of DNA between two strains by means of bacteriophage. Transduction was first demonstrated the early 1960s in *Lactococcus* by Elliker and coworkers,[159,160] where tryptophan independence and streptomycin resistance, respectively, was transferred by a virulent bacteriophage c2. In the 1970s McKay et al. showed transduction of chromosomal traits like maltose and mannose utilization as well as plasmid-linked traits like lactose utilization and proteinase activities using induced temperate phages for the transduction.[161,162] High-frequency transduction of lactose metabolism was shown by McKay et al.[163] in repeated transduction experiments. However, this was not a general phenomenon of plasmid transfer, since pAMβ1 was not transduced with high frequency in secondary transductions.[164]

Birkeland and Holo[165] showed that carrying of the cohesive ends from the temperate bacteriophage ϕLC3 increased the transduction efficiency of plasmids approximately

1000-fold. Recently, Chandry et al.[166] showed that the cos-site from the lytic phage sk1 also increases the transduction frequencies of plasmids.

In addition to lactococci transduction has been demonstrated in *Lb. gasseri* ADH[167] and in *S. thermophilus*.[168] Heller et al.[169] demonstrated transduction in a fermented milk environment by *S. thermophilus* phages.

Since the development in the early 1980s of transformation systems for LAB, there has been only limited interest in transduction. This is probably due to the expected limitations in host range of the temperate and virulent bacteriophages. However, host range (e.g., plaque formation) is not limiting for gene transfer by phage.[169,170]

3. Conjugation

During conjugation, DNA is transferred from the donor cell to the recipient by direct cell-to-cell contact. In gram-negative bacteria this contact can be mediated with structures called sex pili, but they apparently have no role in conjugation between gram-positive bacteria, such as LAB. Instead, sex pheromones, or substances produced by recipient cells promoting the synthesis of a cell aggregation factor by the donor, thus leading to the formation of donor-recipient pairs, are well known, especially among the enterococci.[171] Pheromones, however, are not universal among gram-positive bacteria with conjugative genetic elements. Irrespective of the mechanisms of achieving cell-to-cell contact, the conjugative genetic elements must have certain highly conserved common structures, such as the origin of DNA transfer (*oriT*) and the various genes involved in the actual transfer event (*tra*).

The importance of conjugation among LAB has been indicated in previous chapters in discussions of conjugative transposons and antibiotic resistance plasmids as well as the association between conjugation and group II introns. Among the metabolic plasmids, the conjugative transfer of lactococcal lactose fermentation plasmids has been well reported (see Ref. [172] for review). In *Lc. lactis* 712 the sex factor causing a high frequency of recombination has been thoroughly analyzed.[173,174] In addition to genes involved in the actual DNA transfer, the element contains a group II intron and a gene causing a "clumping" phenotype (*cluA*) associated with the high incidence of conjugation. The sex factor is also associated with an *ISS1*-type insertion element enabling its change of location from chromosome to plasmids.

An important aspect of conjugation is the mobilization of normally nonconjugative plasmids by functional sex factors and conjugative genetic elements. For example, the enterococcal plasmid pAMβ1, which, as noted in Sec. III.E, can be conjugated to several species and genera of LAB, is able to efficiently induce the conjugative transfer of proteinase plasmids in lactococcal hosts.[175]

B. In Vitro Gene Transfer Techniques

Despite the usefulness of in vivo methods available for genetic studies, development of efficient transformation techniques has been necessary for actual gene cloning in LAB. The first efficient methods were based on protoplast transformation, but they have been replaced by electroporation during recent years.

1. Protoplast Transformation

Preparation and regeneration of lactococcal protoplasts was achieved by Gasson,[176] who also demonstrated PEG-induced protoplast fusion and the recombination of both

chromosomal and plasmid-linked markers in the regenerated fusion products. Actual PEG-induced protoplast transformation of lactococci was reported by Kondo and McKay[177] using lactose fermentation plasmids. Subsequently, the transformation protocols were optimized in different laboratories with respect to protoplast regeneration, PEG concentration and molecular weight, PEG treatment time, protoplast concentration, ionic composition of transformation buffers, and bacterial growth phase before protoplasting.[178–180] With optimized methods and using different antibiotic-resistance markers, transformation frequencies of 10^4–10^6 transformants per microgram of DNA were achieved, allowing for the shotgun cloning of both plasmid-linked[178] and chromosomal lactococcal genes[181] in lactococcal hosts.

2. Electroporation

In electroporation, a short electric impulse is conducted through the cell suspension, permeabilizing the cell wall to DNA for a few nanoseconds. This is sufficient for efficient DNA transfer and often for rather high transformation frequencies. The first electroporation of *Lc. lactis* was reported by Harlander[182] and of *Lb. casei* in the same year by Chassy.[183] The electroporation techniques have since then been introduced and optimized for most of the important LAB species and genera such as lactococci, *S. thermophilus*, *Leuconostoc*, and *Lb. plantarum*.[184–188] Recently the method has also been optimized for *Lb. delbrueckii* subsp. *bulgaricus*, which was previously very difficult to transform.[189]

C. General Genetic Recombination and Recombination-Deficient Mutants

In general recombination the DNA introduced into the host may be integrated either with the host chromosome or plasmid, provided that the incoming DNA is sufficiently homologous with the host sequences. The mechanisms involved have mainly been studied using the lactococcal integration vectors as models (see sect. VI). Homologous sequences for these plasmids have been derived either from lactococcal chromosome[190] or from a resident prophage.[191] Amplification of integrated plasmids seems to be a regular phenomenon in these experiments when selective pressure (the presence of an antibiotic against which the inserted plasmid confers resistance) is applied.

Recombination-deficient lactococcal mutants are known. The first of these was obtained by Anderson and McKay[192] after mutagenization with methyl methanesulfonate (MMS). Transfer of plasmids into this strain occurs normally, but introduction of chromosomal markers is inhibited. The central gene controlling bacterial recombination, *recA*, is highly conserved among different genera of both gram-negative and gram-positive organisms, and this has allowed the polymerase chain reaction (PCR)–based cloning of the lactococcal *recA* gene and generation of new *recA* mutants by insertional inactivation.[193,194] These mutants are sensitive to different DNA-damaging agents as well as to oxygen and heat,[195] indicating a global role for RecA in various cellular processes.

VI. TOOLS FOR THE GENETIC MODIFICATION OF LAB

The efficient gene transfer mechanisms, together with the development of cloning vectors, have made the application of recombinant DNA techniques possible for most important LAB. The vectors available range from simple general cloning vectors to constructs

designed for special purposes such as integration or efficient expression of cloned genes. Food-grade vectors devoid of antibiotic resistance markers represent a special case in respect to eventual applications.

In addition to cloning, present-day genetic techniques allow for targeted mutagenesis as an alternative to traditional random mutagenesis of strains by chemical mutagens or radiation.

A. Cloning Vectors

1. General Cloning Vectors

The general cloning vectors typically consist of a plasmid replicon and a marker gene, usually coding for antibiotic resistance, allowing for selection. With LAB the commonly used vectors are based either on cryptic lactococcal or lactobacillar plasmids or derived from large conjugative plasmids such as pAMβ1 and pIP501.[196]

The two most common lactococcal vectors, pGK13 and pNZ12, are based on nearly identical cryptic plasmids pVW01 and pSH71. As noted in Sec. III.A, these plasmids replicate by the rolling circle mechanisms and have a wide host range, also outside LAB. This property makes them very attractive for research purposes, but is a cause of concern in the genetic containment of eventual genetically modified strains in their applications. Also, the accumulation of single-stranded DNA might lead to genetic instability when foreign DNA is inserted into the constructs.[197]

Plasmid pIL253[198] is a deletion derivative of pAMβ1 with a high copy number. It has lost the conjugative properties and does not replicate in gram-negative hosts (in contrast to pVW01- and pSH71-based vectors). This plasmid replicates by the theta mechanism, and the genetic constructs in this background are considered more stable than the ones based on plasmids with the rolling circle type of replication.

By choosing and combining different replication regions functional in different hosts, it is possible to create families of shuttle vectors able to replicate and express selection markers in widely different hosts such as lactococci, lactobacilli, *E. coli*, and *B. subtilis*.[199]

2. Integration Vectors

Integration vectors for LAB have been used for several purposes—mainly for the generation of mutations, either as a random process to identify genes or by site-specific integration gene disruption or gene replacement, in order to mutagenize a specific gene. A large number of vectors have been developed, but only a few can be mentioned within the scope of this review. In addition, site-specific integration vectors based on phage-integration elements allowing stabilization of genes on a specific site in the chromosome have been developed.

Some of the first integration vectors used in LAB (e.g., *Enterococcus*) were derived from transposons like Tn*917* or Tn*916*, both of *Enterococcus* origin. Clewell and coworkers identified Tn*917* in *E. faecalis* DS16 in the late 1970s.[200] The integration of Tn*917* into the target is induced by the presence of small non-inhibitory amounts of erythromycin.[201] Tn*917* has been a workhorse for generating mutations in a large number of gram-positive bacteria, first by Clewell and coworkers for the characterization of hemolysin and conjugation in *E. faecalis*.[202–205]

Subsequently, Tn*917* became a powerful tool for generating mutations in *B. subtilis* by first combining Tn*917* with a temperature-sensitive derivative of the pE194 repli-

con,[206] then introducing a pBR322 replication origin allowing for fast identification of flanking regions of the Tn*917* insertion site,[207] and finally by introducing a promoterless *LacZ* gene to the end of Tn*917* for the identification of promoters.[208] These genetic tools have been utilized for generation of mutations in *Lb. plantarum*,[209] for promoter screening in lactococci,[210,211] and recently for the identification of secretion signals in lactococci.[212]

Clewell and coworkers also identified Tn*916* from *E. faecalis*,[142] and it has been shown that cloned fragments containing integrated Tn*916* can be precisely excised in *E. coli*.[213] Due to its conjugative nature, it has been used as a genetic tool to generate mutations in both gram-positive and gram-negative bacteria.[214] In industrial LAB, Tn*916* and related Tn*918* and Tn*919* have, however, only found limited use, probably because of reported hot-spot integrations.[215] In certain *Lactococcus* strains, including MG1363, Tn*916* has also been reported not to be able to conjugate due to the limited excision and the lack of a host factor for conjugation.[216]

Gene disruption and gene displacement vectors dependent on homologous recombination between cloned fragments (possibly mutagenized) and the chromosome must have conditional replication functions in the strain that should be mutagenized, either by not having functional replication (e.g., vectors with an *E. coli* replicon) or a defective or thermosensitive replicon. This approach was first applied in LAB using replicons from plasmids pBR322 or pE194 with a selectable marker.[190,191]

Later the lactococcal broad host-range plasmid pWV01 was developed to pINT/pORI vector series by eliminating the replication initiation gene *repA* but leaving the replication origin intact. Such a vector could be propagated in strains that contain the repA gene in trans.[217] A drawback of these types of suicide vectors is that they require high transformation frequencies to obtain integration in sufficient numbers of sites for the purpose of randomized mutation. However, by introducing the library into a strain containing the plasmid pVE6007[218] coding for a thermosensitive RepA at its permissive temperature (30°C), followed by growth at the nonpermissive temperature (and subsequent curing of pVE6007), a sufficient number of transformed cells was obtained.[219] By introducing pVE6007 again, a high excision rate was obtained, allowing for rapid analysis of the chromosomal insertion site.[219] The original pORI vectors were later improved by introducing a *lacZ*, which allows the detection of excision in the absence of an easy screening phenotype.[220] A food-grade version using the sucrose gene from *P. pentosaceus* as a screening marker has been developed.[221] Although suicide vectors have been used for random mutagenesis, their main use has been to generate specific gene disruptions or gene-replacement mutations.

Thermosensitive vectors have been derived from pWV01 by mutagenizing the plasmid and screening for thermosensitive plasmid derivatives (e.g., pVE6007) (see above).[218] It was demonstrated that at the nonpermissive temperature chromosomal integration could be selected for. This thermosensitive replicon was later developed into the so-called pG^+ host vector series containing different selectable markers[222] and has been a useful tool for generation of gene-replacement mutations in a number of mesophilic and some thermophilic LAB.

In many thermophilic LAB the pG^+ host vectors and the pINT/pORI two-plasmid system cannot be used because the permissive temperature is too low for sufficient growth of the transformants. The broad host-range cloning vector pSA3, containing the replication origins of the pIP501 and pBR322,[223] has in *Lb. helveticus* been found to be thermosensitive at 45°C[224] and used to generate gene-replacement mutants in this host. Russell and

Klaenhammer[225] observed that the pWV01 wild-type replicon is thermosensitive at elevated temperatures (e.g., 43°C) and adapted the pINT/pORI two-plasmid integration system described by Law et al.[219] to *Lb. acidophilus* and *Lb. gasseri.* Also, a new thermosensitive integration vector for *Lb. gasseri* has been developed, based on a replicon from a cryptic *Lb. curvatus* plasmid pLC2, which proved to be thermosensitive.[226]

As mentioned above, suicide thermosensitive vectors, dependent on homologous recombination between randomly cloned small chromosomal fragments and the chromosome, have been used for random mutagenesis.[191,219] However, vectors used for random integration mutagenesis in industrial LAB have in most cases been based on the random integration of IS elements. As in the case of gene disruption and displacement vectors, these vectors should have restricted replication in the host.

Romero and Klaenhammer[227] constructed a composite transposon on a suicide vector based on two IS946 elements flanking the *cat* gene from pC194, which allowed for selection of integrant by chloramphenicol. Polzin and McKay[228] utilized a natural thermosensitive plasmid replicon from pSK11L from *Lactococcus* to develop an IS981-based integration system and a selectable erythromycin marker. A non-replicating vector containing an the ISS1 element containing an *ery* gene as selection marker was used to generate a physical and genetic markers in *Lactococcus*.[229] In *Lb. gasseri* the IS1223 was rescued on pSA3. Subsequently the gram-positive replication origin was deleted, and the derived vector pTRK327 was used for generating random mutations in *Lb. gasseri*.[230] However, neither of the above-mentioned vectors have gained wide use. The frequently reported IS vectors used for random mutagenesis are derivatives of the pG^+ host vectors carrying the ISS1 element.[136] This is probably due to the wide host range of the temperature-sensitive pWV01-derived replicon. One advantage of the last-mentioned system is that vector integrants can be excised, leaving behind only an extra copy of the IS element. This technology can therefore generate (random) food-grade mutants in a two-step process, which can be applied in foods or in food models.

Site-specific integration vectors have also been developed based on the temperate phage integration systems from LAB. These vectors will integrate cloned genes in one copy on a specific location in the chromosome, allowing for stabilization of heterologous or plasmid-encoded genes. Their main use so far has been to study regulation of promoters using reporter genes, where their single copy mimics the chromosomal situation. The first of these integration vectors was based on the adh phage integrase gene and the corresponding *attP* site cloned onto pSA34, a pSA3 derivative without the gram-positive replication origin.[231] Lillehaug and Birkeland[232] described the site-specific integration in *Lactococcus* using ϕLC3 integrase and *attP* site. This was later developed into an integration vector pINT2 generating stable one-copy integration.[233]

From the lactococcal phage TP901-1, site-specific integration was demonstrated.[234] It was later reported that the integration frequency of this resolvase-like integration system was unusually high, allowing for integration into strains with low transformability.[235] Transcriptional fusion integration vectors derived from the TP901-1 integration system were developed with the *gusA* and *lacLM* as reporter genes.[236] Later a version with the *pipI* gene from *Lb. helveticus* was obtained (P. Varmanen, personal communication). Recently, Stoll et al.[237] showed that the TP901-1 integration system under the right conditions could be applied to *E. coli* and even to mammalian cells, indicating that the TP901-1 integrase system does not require any specific host factors except the *attB* site.

The integrase system of the *Lb. delbrueckii* phage mv4 may find special application in LAB because the mv4 site specifically integrates into the end of a $tRNA^{ser}$ without

disrupting the tRNA molecule.[238] It was first shown to be able to integrate site-specifically into the corresponding tRNA in *Lb. plantarum*,[238] and later into a number of LAB.[239]

3. Expression Vectors

Overexpression of homologous or heterologous genes can be achieved by a fusion of a strong or regulated promoter to the gene of interest. In addition, it is often necessary to add signal sequences that allow export of the expressed gene product. In case the expressed protein is to be displayed on the surface of the cell (e.g., for vaccine production), a cell wall or membrane anchor may also be necessary.

Many promoters have been identified from LAB either by direct screening (e.g., Refs.[211,240–244]) or as a result of the analysis of specific genes (e.g., Refs.[245,246]). The first expression vectors utilized strong constitutive promoters, and a number of such vectors have been constructed with different promoters from different LAB. Common for them is that the expressed proteins are normally found in the cytoplasm as biologically inactive in inclusion bodies (e.g., Ref.[247]) as a result of the high expression and accumulation of protein. Also, cloning problems may occur if the gene product is toxic to the cell at the expressed concentration (e.g., Ref.[248]). Both of these problems can be avoided by choosing vectors with natural promoters having different strengths. Although developed for a different purpose, a series of synthetic constitutive promoters that allow the modulation of the expression over a 2000-fold range may be useful for fine-tuning of gene activity.[249]

Another way to reduce problems with inclusion bodies or in cloning and expression of toxic gene products is to use a regulated promoter that allows for optimization of expression. Several promoters have been suggested for this purpose, including phage-derived thermoinducible promoters based on genetic switches of temperate phages (e.g., Refs.[250,251]), middle phage promoters that can be regulated by phage infection,[252] sugar-regulated promoters,[253–257] environmentally regulated promoters such as pH,[246] or NaCl-induced promoters.[258] So far only the nisin-inducible promoter, P_{nisA}, for nisin A production[259] has found widespread use. The P_{nisA} promoter has been cloned on the wide host range replicon from pWV01 in different versions.[260] This system, called NICE, was developed by the NIZO group in the mid-1990s and relies on the autoregulation of nisin production by nisin itself through the *nisRK* response regulator.[261] Initially the vectors were developed for *Lactococcus*.[260] The *nisRK* regulator was integrated on the chromosome of *Lc. lactis* MG1363 in the *pepN* gene; alternatively, a derivative of Tn7562 containing the whole nisin operon, including the nisin immunity regions but with the structural nisin gene deleted, was conjugated into MG1363. One of the advantages is that the expression level of the P_{nisA} promoter vectors can be fine-tuned by the amount of nisin added and the strain used.[260] Later, a two-vector system was developed in which the *nisRK* genes are located on a separate compatible broad-host-range vector, which allowed nisin-controlled expression in a number of gram-positive bacteria.[262,263] In some cases the two-vector system has been reported to cause problems.[264] These were solved by integrating the *nisRK* on the chromosome using the broad-host-range integration vector pMEC1.[238] Broad host-range single-plasmid nisin-controlled vectors have recently been constructed to simplify the induction system.[265] Interestingly, it has been reported that the P_{nisA} promoter is also induced by galactose and lactose at least in *Lc. lactis* and that this regulation is independent of *nisRK*.[266,267]

Secretion signals are in most cases necessary if the expressed product has to be externalized for easier downstream processing, although Walker and Klaenhammer[268] described secretion of *S. thermophilus* β-galactosidase in *Lc. lactis* by a controlled expression of a Φ31 holin and lysin cassette. However, peptidases were not externalized using this system.[269]

A number of secretion signals have been found by screening of LAB for sequences that are able to export products of reporter genes such as α-amylase, β-lactamase, nuclease, or other gene products easy to screen for.[212,270–272] Others have been found from genes where the gene product is known to be excreted, e.g., the secretion signal sequences for *prtP* and *usp45* from *Lc. lactis*,[273,274] the α-amylase genes from *S. bovis* [275] and *Lb. amylovorus*,[276] and the S-layer protein gene from *Lb. brevis*.[277,278] Addition of the nine-residue synthetic propeptide LEIDDTCDA after the signal sequence was shown to improve the secretion efficiency in *Lactococcus* significantly,[279] while mutation of host genes like *htrA* influences the stability of the expressed and secreted proteins.[280]

Within the last decade the use of LAB as live vaccines has attracted increasing interest (e.g., Refs.[281–284]). Although in most cases the antigen has been excreted to the medium, it may be an additional advantage to be able to transfer the antigen to the surface of the cell. The cell-wall anchor of the *S. pyogenes* M6 protein has been used by several groups for anchoring proteins to the surface of LAB (e.g., Refs.[284–286]). Similar cell-wall anchors from *Staphylococcus aureus* protein A[287] and lactococcal PrtP[281,288] and AmcA[289] have also been used for targeting proteins on the surface.

4. Food-Grade Cloning Vectors

Although consumer attitudes towards genetic engineering, particularly in Europe, but increasingly also in the United States, are skeptical, a number of so-called food-grade approaches to genetic engineering of LAB have been suggested. Some of these are based on the construction of food-grade vectors using food-grade markers, while others have relied on a "clean homologous recombination" approach. Food-grade markers can be divided into two groups: that is dominant selection and screening markers (group 1a and 1b) and complementation selection and screening (group 2a and 2b) markers.[290] The 1a and 2a markers are directly selectable, while markers in 1b and 2b must be screened.

Group 1a is based on genes that give resistance to, for example, a bacteriocin or a heavy metal ion and can therefore be directly selected for in sensitive strains. Among the first genes to be suggested as putative food-grade markers was a nisin-resistance gene, *nsr*. The *nsr* gene was identified by several groups and is unrelated to the nisin immunity gene, *nisI*. It was first found on plasmid pNP40 from *Lc. lactis* DRC3,[291] and was subsequently cloned as pFM011 and sequenced.[292,293] A cloning vector pVS40 was constructed from a related *nsr* gene encoded on plasmid pSF01 from *Lc. lactis* strain 10.088[294] and a *Lactococcus* plasmid origin of replication belonging to the theta group.[58] Both plasmids were shown to be useful for cloning in *Lactococcus* [57,295]

The immunity gene of lactacin F, *lafI*, isolated from *Lb. johnsonii* VPI 11088 was shown to be expressed in a number of *Lactobacillus* species and could be selected for at least in *Lb. fermentum*.[296] The nisin and the lacticin 3147 immunity genes have been suggested as food-grade markers for vector constructions in *Lactococcus*.[297,298]

Both these selection markers have been shown to function outside *Lactococcus.* In the future we will undoubtedly see more food-grade markers based on bacteriocin resistance and immunity genes. Recently it was shown that the *nisRKFEG* may also be used as a food-grade selectable marker, although its size limits its applicability.[299]

Cadmium resistance genes encoded by natural plasmids from lactococci have also been used as a food-grade selectable markers in conjugal transfer and in vector construction.[115,300] Although the selection for cadmium resistance is promising, cadmium at the same time is a heavy metal, and therefore this resistance may not be considered ethical from an environmental point of view and therefore not "food-grade," even though the genes derive from organisms having a long history for being safe.

Group 1b food-grade markers are based on the fermentation of rare or unusual carbohydrates. Due to the variation in carbohydrate fermentation profiles, a number of carbohydrate catabolism genes may be utilized as screening markers in food-grade vectors. Only two examples will be mentioned here. Because the ability to ferment D-xylose is limited to a few species of LAB, the D-xylose catabolism encoding genes of *Lb. pentosus* have been suggested as food-grade markers[301] in a screening for transformants of *Lactobacillus* strains. Similarly, an α-galactosidase gene (*ada*) from *Lc. raffinolactis* could be used as a screening marker for the utilization of melibiose in *Lc. lactis* and *P. acidilactici*.[302] Data indicated that in addition to *ada*, a functional galactose permease gene, *galA*, was also necessary.

Group 2a is based on complementation of mutations in a chromosomal gene essential for growth under certain conditions. It is therefore only possible to conduct the complementation in strains where the corresponding mutation has previously been introduced. The ochre suppressor gene *subB* from *Lc. lactis* was used to construct a food-grade vector pFG1 where a chromosomal auxothophic purine mutation of *Lc. lactis* DN209 was complemented.[303] Later this system was improved in pFG200 using the amber suppressor gene *subD* in combination with amber mutation in *pyrF*,[304] as growth inhibition was seen in industrial strains when the *subB* suppressor was used. The advantage of the suppressor system as a food-grade marker, besides its small size (about 350 bp), is that milk contains low amounts of purines and pyrimidines, causing the vector to be stably maintained in milk fermentations.

Another promising complementation system relies on the essential alanine racemase gene *alr* present in *Lb. plantarum* and *Lc. lactis.* Mutations in the *alr* gene make the strain auxotrophic for D-alanine, necessary for cell wall synthesis. Recently it was shown that *alr* genes from *Lc. lactis* and *Lb. plantarum* heterologously complement chromosomal *alr* mutations in both species.[305] However, the applicability in LAB in general may be questioned as two Alr activities has been observed in *Lb. reuteri*.[306]

Group 2b food-grade markers are based on complementation of some specific mutation in a carbohydrate fermentation operon. Like the 2a markers, they only function in strains where the corresponding mutation has been introduced. No direct selection for such markers is possible, but screening using proper concentration of buffers, indicators, and sugar allows for isolation of transformants. One example of such a marker is the *lacF* gene, encoding enzyme III in the lactose PEP : PTS uptake system. The *lacF* gene was shown to complement a *lacF* deletion on a chromosomally located copy of the lactose operon in *Lc. lactis* FI7794, a derivative of MG5267.[307] The advantage of this marker is its small size. The *lacF* marker has been used in the construction of a number of food-grade vectors using the regulated nisin promoter.[308] Also, *lacG* from *Lb. casei*, encoding phospho-β-galactosidase in the lactose operon, has been used in a similar approach.[309]

VII. APPLICATIONS OF GENETICALLY MODIFIED LAB

While recombinant DNA techniques and the general developments in molecular biology have substantially increased our knowledge of the fundamental genetic aspects of LAB, the practical applications have been somewhat slow to materialize. One of the main reasons for this is the complicated nature of food fermentations, where the roles of single genes and their relative activities in the desired properties of the end products are still poorly known. In addition to the improvement of the present starter strains and products based on them, completely novel applications of LAB may be accomplished in the near future. Metabolic engineering and the use of LAB as vaccines or immunotherapeutic agents are examples of new applications attracting much attention.

A. Examples of Metabolic Engineering

To reconstruct metabolic pathways from genome data is a formidable task made possible by the recent advances in molecular genetics. Lactococci were the first LAB in which metabolic engineering studies were developed due to the large body of available data on their genomic structure and relatively well-known physiology (reviewed in Refs.[310–312]). The first example was a nearly complete redirection of the metabolic flux by the inactivation of the enzyme lactate dehydrogenase in *Lc. lactis* by genetic disruption of the relevant gene.[313] The manipulated clone performed a mixed acid fermentation under anaerobic conditions, while in the presence of oxygen it produced almost entirely acetoin.

Diacetyl production of up to 50% of available sugar was realized by overexpressing *S. mutans* NADH oxidase in *Lc. lactis* (leading to less lactate and enhanced acetoin production) in combination with a genetic disruption of *aldB*, the gene for α-acetolactate decarboxylase.[314]

B. LAB as Immunotherapeutic Agents: Genetic Aspects

The possibility of introducing heterologous antigens into LAB, to be used as delivery vehicles of foreign epitopes is an exciting and rapidly evolving application of genetic engineering.[315] LAB possess a number of properties that make them attractive candidates for oral vaccination (general safety, traditions of food use, technological advantages, etc.) An especially attractive feature is the ability of some LAB to adhere to the intestinal epithelium and to persist, and even reproduce themselves, in the human gut. In the following paragraphs we discuss the most recently obtained results in this field, concentrating on the genetic aspects (rather than the immunological details) of this potential application.

Organisms to be used as oral vaccines are designed for expressing foreign epitopes on their outer surfaces, and special expression and secretion vectors have been constructed. The use of a surface display system based on the *PrtP* sequence was first used to express tetanus toxin fragment C (TTFC) in cells of *Lc. lactis*.[281] This organism has been widely experimentally exploited for vaccination purposes.[316,317] However, no consistent picture of its final applicability has emerged.[318]

Lactobacilli have also been used to act as vaccine delivery vectors,[319] and TTFC, again, is the most studied antigen.[320] In contrast to *Lc. lactis*, in which the studies conducted so far have used only the strain MG1363 or its derivatives, a number of lactobacilli have been tested for antigen delivery. Genes under control of *xyl*R or *cbh* promoter of *Lactobacillus* were initially used,[321] but inconsistent results were obtained. Subsequently both successful intracellular and the cell surface expression of TTFC has been achieved by means

of specially developed expression vectors, based on secretion signals of amylase or peptidase enzymes.

Immunological results obtained using animal models have shown the relevance of the genetic construction to obtain good immune response in the host. A number of plasmid expression vectors have been designed for *Lb. plantarum* NCIMB 8826 based on *ldh* constitutive promoter and transcriptional termination signals. Different localizations of the antigen (intracellular, secreted, or surface-exposed) were obtained,[322] leading to different immunological responses.

A model antigen, the cell wall-anchored proteinase PrtB of *Lb. delbrueckii* subsp. *bulgaricus*, has also been used to evaluate the possibility of using a strain of *Lb. johnsonii* to act as vaccine-delivery vehicle. Results showed that this recombinant *Lactobacillus* can induce both systemic and local mucosal immune responses.[323]

Another approach for providing disease protection by recombinant lactobacilli has been the cloning in *Lb. zeae* of an antibody fragment recognizing a streptococcal antigen typical to cariogenic *S. mutans.* Administration of these bacteria to a rat model of dental caries resulted in the depression of both *S. mutans* counts and caries development.[324]

In addition to antigens and antibodies, the signal molecules of the immune system have a decisive role in immunological and inflammatory reactions. *Lc. lactis* designed to express IL-10 on its surface has been successfully used to treat experimentally induced murine colitis.[325] This finding could open new possibilities for the development of next-generation probiotic strains designed to specifically treat certain diseases or disorders.

VIII. SUMMARY

Genomic sequences of the most important LAB species are increasingly becoming available, while the research on proteomics is starting to take advantage of the new possibilities of the accumulating sequence data. At the same time recombinant DNA techniques have become even more sophisticated, and even difficult species are becoming amenable to genetic modification.

It is to be hoped that the rapidly accumulating genetic information will enhance our understanding both of the basic physiology of LAB and especially of their role in food fermentations at the metabolic level. This information is essential if the traditional uses of LAB are to be optimized using genetic techniques. The prospect of engineering the metabolic pathways is also becoming more real.

The development of genetically modified LAB for completely new applications, such as vaccines or designed probiotics, is a challenging prospect. In addition to technical and scientific problems, the unanswered question of public acceptance of these types of products (as well as the position of regulatory bodies) has to be taken into account. Nevertheless, the future of genetic modification of LAB may well be in their health-related uses.

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8

Bacteriophage and Antiphage Mechanisms of Lactic Acid Bacteria

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I. INTRODUCTION

Food fermentations rely on actively growing lactic acid bacteria (LAB) which either are added as starter cultures or grow spontaneously in the food matrix. The fermentation capabilities of lactic acid bacteria can be severely inhibited by a panoply of nonviral inhibitors, such as bacteriocins, nisin, residues from antibiotic or disinfectant treatments, or the lactoperoxidase-thiocyanate-hydrogen peroxide system present in raw milk. However, the major commercial problem results from bacteriophage infections. These bacterial viruses were identified as filter-transmissible agents by the work of Twort in 1915 and d'Hérelle in 1917.[1] Their submicroscopic sperm-like morphology remained undetected until the first electron microscopes became available during the 1940s. Phages attacking *Lactococcus lactis* have been known since the 1930s, when Whitehead and Cox in New Zealand observed that phages were responsible for the failure in the acid-producing activity of a cheese starter culture.[2]

In the area of food fermentation, the permanent threat of phage infection is particularly manifested in the dairy field. Here, phage attacks on lactic acid bacteria during the fermentation process result in an unacceptably low production of lactic acid and flavor compounds along with reduced proteolysis. Thus, starter activity is either severely affected ("slow vats") or, in extreme cases, a complete failure of starter growth may occur ("dead vats"). Due to the constant risk of economic loss, phage control is a major area of concern in handling lactic acid bacteria as starter cultures. Phages have also

been isolated from other fermentations, e.g., in the production of sauerkraut, coffee, and wine, but most often these attacks are not as destructive as in milk fermentation. These worldwide efforts focus on the understanding of the complex and dynamic mechanisms of phage-host relationships, including (a) the characterization of the phage population involved, including genomics analysis, (b) the analysis of "natural" and "intelligent" bacterial systems of phage defense, and (c) the identification of phage counterdefense mechanisms.

II. BACTERIOPHAGES

A. Phage Morphology

All phages known from lactic acid bacteria belong to the order Caudovirales, meaning that they are tailed phages. Tails and heads are the main structural components of phages, and their sizes and structures are the basis for establishing various morphotypes. LAB phages are members of the three morphotypes A (contractile tails), B (long, noncontractile tails), and C (short noncontractile tails) according to the classification scheme of Bradley.[3] Today they are represented as the phage families Myoviridae, Siphoviridae, and Podoviridae, respectively.[4,5] These three families are further differentiated in phages displaying isometric, small prolate, or large prolate (elongated) heads (morphotypes 1–3, respectively). Thus, the isometric-headed *Streptococcus thermophilus* phage shown in Fig. 1 is a member of the Siphoviridae of morphotype B1. The electron micrographs of lactococcal phages (Fig. 2) illustrate further examples of phages from the Siphoviridae and the Podoviridae families. Most characterized LAB phages belong to Siphoviridae

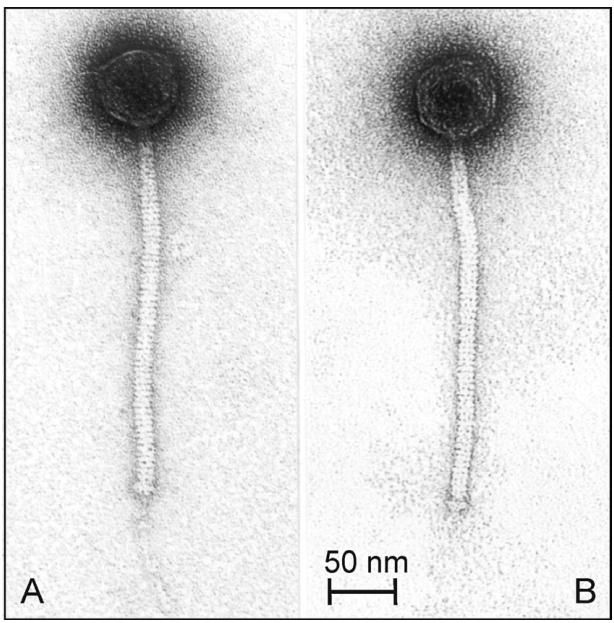

Figure 1 Electron micrographs of a virulent bacteriophage of *Streptococcus thermophilus* with (A) and without (B) a single tail fiber.

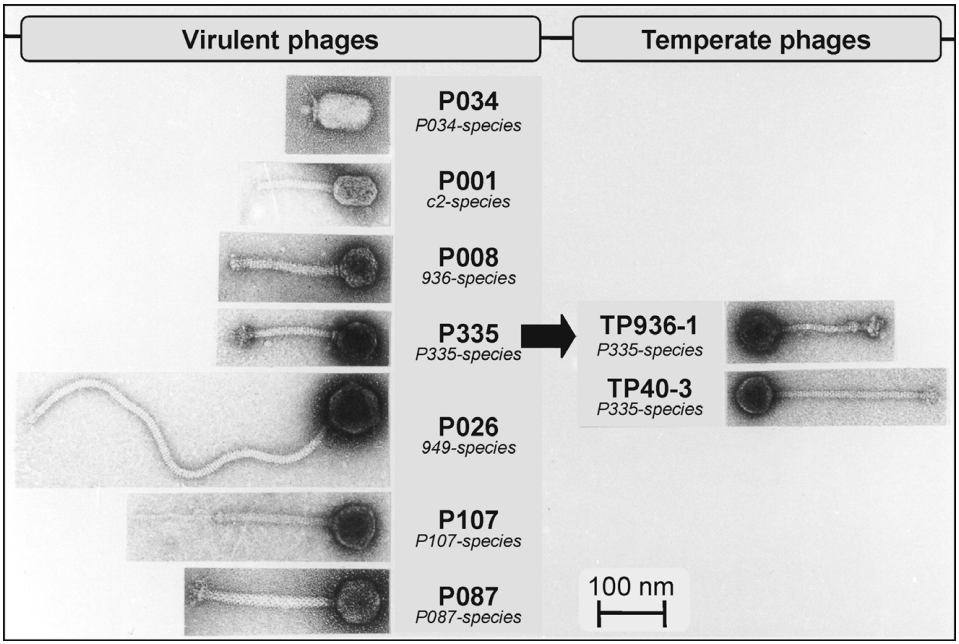

Figure 2 Transmission electron micrographs of the various morphotypes of lactococcal phages from one collection (modified from Ref.[13]). The phage taxonomy from Jarvis et al.[6] is indicated. The arrow highlights the close genetic relationship of virulent and temperate phages of the P335 species.

B1 morphotype and only phages homologous for *Lactococcus* and *Lactobacillus plantarum* morphotype C3 Podoviridae have been isolated. Morphotype A1 Myoviridae have been observed for *Lactobacillus helveticus*, *Lactobacillus plantarum*, and *Lactobacillus fermentum*. Further differentiation of LAB phages into phage species was originally based on morphology, serology, DNA size and structure, DNA homology, and, within the last decade, on complete phage genome sequences.

B. Phage Classification

1. *Lactococcus lactis* Phages

Lactococcal phages have been differentiated into 10 (originally 12) phage species, each represented by a type phage.[6] Phage isolates belonging to all except one species (i.e., species P335) are strictly virulent. The majority of phages belong to the Siphoviridae family; only two belong to the Podoviridae. Isometric-headed phages of B1 morphotype are divided into seven phage species differing in tail length and structure. Members of species 936 are the most numerous, followed by the P335 species. Species P107, P087, 1358, BK5-T, and 949 are either unique or include only a few phages. Recently it was suggested to merge the phage species BK5-T and P335.[7,8] The prolate-headed phages have three morphotypes, B2 (prolate head, long noncontractile tail), C2 (prolate head, very short tail), and C3 (elongated prolate head, very short tail), which are grouped into the species c2, P034, and KSY1, respectively. Isometric-headed phages belonging to

the 936 species are the predominant lytic phages isolated from dairy plants in Australia, New Zealand, the United States, and Europe; however, in Canada prolate-headed phages belonging to species c2 were temporarily found to be the predominating species.[9] Within the last decade lytic P335 phages have appeared in dairy plants.[10–12] An example illustrating the various morphotypes found in one phage collection is shown in Fig. 2.[13]

2. *Lactobacillus* Phages

Mainly virulent phages homologous for species of *Lactobacillus* involved in milk fermentations have been isolated. These include phages for *Lb. delbrueckii* subsp. *lactis* and *bulgaricus*, *Lb. helveticus*, *Lb. acidophilus*, *Lb. gasseri*, *Lb. casei*, and *Lb. rhamnosus*. They all have phages that belong to morphotype B1 Siphoviridae.[14,15] Virulent and temperate B1 Siphoviridae *Lb. casei* and *Lb. rhamnosus* phages are genetically related.[14,15] The temperate phages ϕFSW and A2 are the best studied *Lb. casei* phages today.[16,17] Within phages for *Lb. delbrueckii* subsp. *bulgaricus* and subsp. *lactis* phages both B1 and B3 Siphoviridae morphotypes have been observed. These phages are classified into the four DNA homology groups *a*, *b*, *c*, and *d*, of which *a* is the most numerous.[18,19] Interestingly, most phages for *Lb. helveticus* belong to the A1 Myoviridae family with either a short (160 nm) or a long (260 nm) contractile tail.[20] The temperate *Lb. acidophilus* phage ϕy8 exhibits the unusual B3 Siphoviridae morphology with an elongated head, which apparently is widespread in U.S. yogurt production.[21] In general, the DNA homology between the lytic and temperate phages is much higher for phages of *Lactobacillus* than for lactococcal phages.[22,23]

It seems that the morphotype of the nondairy phages varies more than that of dairy phages. Thus, the lytic and temperate phages of *Lb. plantarum* belong to all three morphotypes (B1 Siphoviridae,[24,25] A1 Myoviridae,[26] and C1 Podoviridae[27]). Unusually large genome sizes have been reported for phage representatives of the first two morphotypes.[24] Similarly, *Lb. fermentum* phages belong to the A1 Myoviridae[28] and to B1 and B3 Siphoviridae.[29] *Lb. sake* phages (B1 Siphoviridae) have been isolated from fermented sausage.[30]

3. *Streptococcus Thermophilus* Phages

All virulent and temperate *Streptococcus thermophilus* phages have the same basic morphology (B1 Siphoviridae, as shown in Fig. 1)[31–33] and share significant DNA homology. Lysogeny is a rare event in *S. thermophilus*,[34–38] and only 1–10% of the *S. thermophilus* strains screened in different collections were reported to be lysogenic. *S. thermophilus* phages are distinguished into two groups on basis of the mode of DNA packaging and the pattern of the structural proteins.[39] Representatives of both groups have been proposed as reference phages for phage taxonomy (phages Sfi21 and Sfi11, respectively).[40]

4. *Leuconostoc* and *Oenococcus* Phages

Lytic *Leuconostoc* (*Ln. mesenteroides*, *Ln. fallax*) phages have been isolated from different industrial fermentations, including coffee, sauerkraut, and dairy foods. Phages isolated from sauerkraut were assigned to the B1 Siphoviridae and A1 Myoviridae families and were genetically distinct, as shown by randomly amplified polymorphic DNA fingerprinting.[41] Phage of *Lc. mesenteroides* derived either from coffee or from dairy fermentations were differentiated into six groups with no significant DNA homology.[42] *Leuconostoc*

phages isolated from dairy fermentations[43] were found to be genetically related and showed a typical B1 Siphoviridae morphology as did phages isolated earlier.[44–46]

Lysogeny has not been studied for these bacteria but is common for *Oenococcus oenos* used for malolactic fermentations in wine.[47,48] Approximately one half of *Oenococcus* strains harbor Siphoviridae prophages.[49] *Oenococcus* temperate phages share DNA homology and have been classified into α- and β-subgroups.[50]

5. *Pediococcus* Phages

Phages virulent for *Pediococcus acidilactici* are unknown today, but have been described for halophilic pediococci used for soy sauce fermentation.[51] Temperate *P. acidilactici* phages isolated recently were shown to belong to two unrelated DNA homology groups and were B1 Siphoviridae members.[52]

C. Phage Genomics

Phages infecting lactic acid bacteria contain double-stranded DNA in a linear form in the phage head. In principle, the G + C content of the phage genome is similar to the G + C composition of the bacterial hosts' chromosomes, reflecting the intimate phage/host relationship; it is 36% for phages of *Lc. lactis*[6] and 45% for phages of *Lb. casei*.[17] The amount of phage DNA to be packed in the interior of the phage head is limited by the head size. Genome sizes usually vary from 18 to 55 kb,[6] although sizes up to 134 kb have also been measured for a few lactococcal phages.[53] According to their mode of packaging (see below), phage genomes have either cohesive (single-stranded) ends (*cos*-type phages) or terminally redundant termini (*pac*-type phages).

The first complete phage genome sequence analyses were performed for lactococcal phages bIL67[54] and c2,[55] respectively. Today, genome sequences of several phages, both lytic and temperate phages from *Lactococcus, Lactobacillus*, and *Streptococcus thermophilus*, have been published (Table 1). Analyses of these data give important information on genome organization, phage homology, and phage evolution. The phage-encoded genes are arranged in a compact form, and overlapping of genes is common. Phage genes with related functions are clustered in temporarily expressed functional segments (modules).[56,57]

1. *Lactococcus lactis*

Representatives for each of the three main species of lactococcal phages have been sequenced (Table 1; Fig. 3). The genome sizes of the c2-like phages are shorter than those of the 936-like phages and of the P335 phages. On the relatively small genome of the *cos*-type phage bIL67, 37 open reading frames (ORFs) were organized in two divergent clusters, representing the early (genes for DNA replication and recombination) and late (genes for structural proteins, terminase, lysis genes) regions.[54] The sequence of the prototype prolate-headed phage c2 shared approximately 80% nucleotide sequence identity with the bIL67 sequence.[55] The organization of the two genomes was conserved; differences were found for three minor structural genes and the putative tail fiber/adsorption protein.

Complete genome sequences are available for two virulent phages of the lactococcal 936 species. Fifty-four and 64 ORFs are present on the genomes of the *cos*-type phages sk1[58] and bIL170,[59] respectively. Their genomic organization is characterized by a large late gene cluster covering nearly half of the genomes, which encompass genes

Table 1 Phages and Prophages of Lactic Acid Bacteria with Completed Genome Sequence Analysis

Host/phage	Life cycle	Species[a]	Genome size (kb)	Genome type	Ref.
L. lactis					
bIL67	Virulent	c2-like	22.195	*cos* site	54
c2	Virulent	c2-like	22.172	*cos* site	55
sk1	Virulent	936-like	28.451	*cos* site	58
bIL170	Virulent	936-like	31.754	*cos* site	59
P482	Virulent	936-like	30.945	*cos* site	See footnote(1)
BK5-T	Temperate	BK5-T-like	40.003	*cos* site	60
r1t	Temperate	P335-like	33.350	*cos* site	61
Tuc2009	Temperate	P335-like	38.347	*pac* site	62
TP901-1	Temperate	P335-like	37.667	*pac* site	63
ul36	Virulent	P335-like	36.798	n.r	8
bIL285	Inducible prophage	P335-like	35.538	n.r.	7
bIL286	Inducible prophage	P335-like	41.834	n.r.	7
bIL309	Inducible prophage	P335-like	36.949	n.r.	7
bIL310	Inducible prophage	n.r.	14.957	n.r.	7
bIL311	Noninducible prophage	n.r.	14.510	n.r.	7
bIL312	Inducible prophage	n.r.	15.179	n.r.	7
4268	Virulent	P335-like	36.596	n.r.	See footnote(2)
S. thermophilus					
Sfi21	Temperate	Sfi21-like	40.739	*cos* site	64
O1205	Temperate	Sfi11-like	43.075	*pac* site	65
Sfi19	Virulent	Sfi21-like	37.370	*cos* site	66
DT1	Virulent	Sfi21-like	34.820	*cos* site	67
7201	Virulent	Sfi21-like	35.466	*cos* site	68
Sfi11	Virulent	Sfi11-like	39.807	*pac* site	69
TP-J34	Temperate	Sfi11-like	45.605	*pac* site	See footnote(3)
Lb. delbrueckii					
LL-H	Virulent	phage group a	34.659	*pac* site	23
Lb. plantarum					
ϕg1e	Temperate	n.r.	42.259	*pac* site	70
Lb. gasseri					
ϕadh	Temperate	n.r.	43.785	*cos* site	71
Lb. casei					
A2	Temperate	n.r.	43.411	*cos* site	17
Lb. johnsonii					
Lj965	Noninducible prophage	n.r.	39	n.r.	57,72
Lj928	Noninducible prophage	n.r.	39	n.r.	57,72
Lj771	Noninducible prophage	n.r.	42	n.r.	57,72

n.r.: not reported

[a]According to Refs.[6,22,57]

(1)C. Glöckner and D. Blohm, unpublished; (2)A. Coffey and P. Ross, unpublished; (3)H. Neve and K.J. Heller, unpublished.

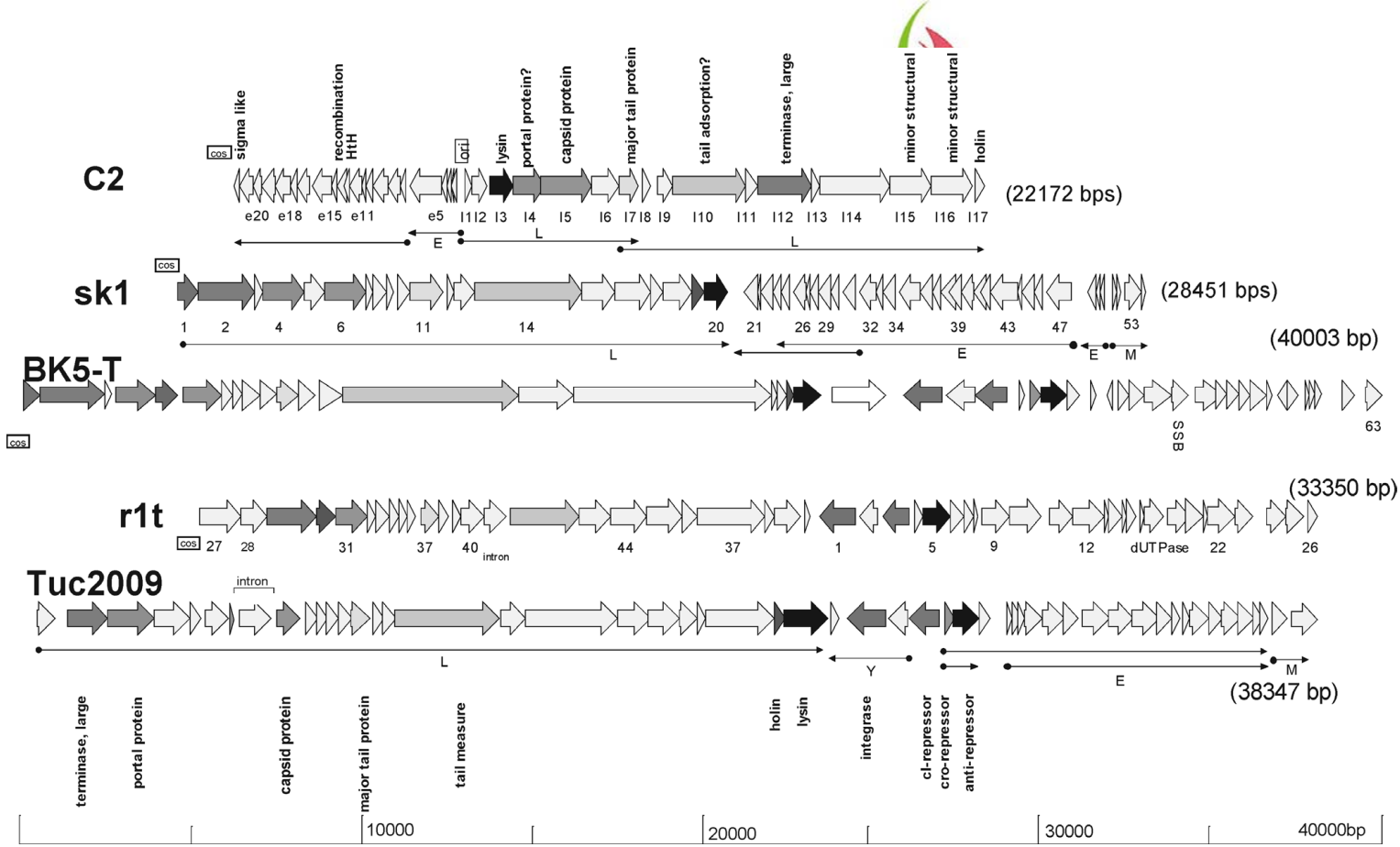

Figure 3 Genome organization of lactococcal phages c2, sk1, BK5-T, r1-t, and Tuc2009. The thin arrows represent transcription maps (E, early; M, middle; L, late transcripts). The same grey shading is used for corresponding genes. (Reprinted from Refs.[56,57] with kind permission of Dr. Harald Brüssow, of Annual Reviews [www.annualreviews.org], and of Kluwer Academic Publishers)

coding for the small and large terminase subunits, the structural proteins, and the lysis genes (length:~16 kb). Early and middle genes are directed towards the late genes. The similarity of the sk1 with the bIL170 genome was greater than 80% on the nucleotide level. Although c2 and 936 phages are distinct phage species with no DNA homology, a significant number of sk1 and bIL170 proteins (i.e., 9 and 10, respectively) share similarity with proteins from the prolate-headed phages.

An impressive number of genome sequences is now available for phages of the P335 species. This species is heterogeneous, and its phages share a limited DNA homology. Comparison of the different phage genomes revealed that these phage genomes are genetic mosaics and that the P335 species should be regarded as a "quasi-species." The phages are either virulent or temperate (see Table 1) and display two different DNA-packaging mechanisms. Their genomes are organized in a life-cycle–specific manner in two oppositely orientated clusters. One of them represents the lysogeny module; the second includes modules for DNA replication, transcription regulation, structural proteins, and host lysis. All P335 phages reveal DNA homology over the lysogeny genes, DNA replication, and putative transcription regulation genes.[68] However, comparison of the DNA sequence homology of their structural genes resulted in the differentiation of distinct phage groups. The structural genes of the *cos*-site phage r1t[61] are unique and share no homology with those of other P335 phages but are related to phage DNA of *Mycobacteria* and of *Streptococcus pyogenes*.[73] The genomes of the *pac*-type temperate phages Tuc2009[62] and TP901-1[63] and of the virulent phage ul36[8] share the highest nucleotide similarity of 40–50% over the whole sequence and showed the highest relatedness within the structural gene cluster. Although ul36 is a lytic phage, its genome contains a complete lysogeny module. Three intact and three truncated P335-type prophages were identified in the chromosome of *Lc. lactis* IL1403.[7] The smaller IL1403 prophages lack genes for phage morphogenesis and cell lysis. The large IL1403 prophages bIl285, bIl286, and bIL309 share homology over 10–33% of their length with other P335 phages (e.g., r1t, Tuc2009). The temperate *cos*-site phage BK5-T,[60] originally placed taxonomically in a distinct lactococcal phage species[6] shares 44% DNA homology with the IL1403 prophage bIL286. These two phages and the IL1403 prophage IL309 have highly similar structural proteins,[68] and it was suggested to merge phage BK5-T with the P335 phages.[8] The DNA packaging and head structural genes of the BK5-T genome have significant homology with the corresponding genomic region of the *cos*-site *S. thermophilus* phage Sfi21.[74] Data show that the mutual homology between 936-like phages or c2-like phages is much higher than between P335-like phages. The high diversity of P335 phages probably results from recombination with prophage or prophage remnants in the chromosome of bacteria.

2. *Streptococcus thermophilus*

Unlike *Lc. lactis* phages, all *S. thermophilus* phages, either virulent or temperate, share DNA homology. Today, genome sequences of two temperate *S. thermophilus* phages (Sfi21,[66] O1205[65]) and of four virulent phages (Sfi11,[69] Sfi19,[66] 7201,[68] DT1[67]) have been published (Table 1). Two completely different late gene clusters covering the DNA packaging, head and tail genes were identified.[39] This division matched with genome topology, i.e., the presence of either *cos*- or *pac*-containing DNA. Accordingly, *S. thermophilus* phages were grouped into two classes: *cos*-type phages (reference phage Sfi21) reveal two main structural proteins, while *pac*-type phages (reference phage Sfi11) have three main structural proteins. The two types of structural gene clusters lack any nucleotide similarity. With respect to genome diversity and conservation,

the *S. thermophilus* genomes were segmented into four parts: besides the DNA packaging/structural gene clusters, three further main segments were identified with different degrees of diversity. Segment 2 covers the putative tail fiber, lysis and lysogeny genes, segment 3 the putative DNA replication module, and finally a segment early genes important for transcriptional regulation.[57] The highest degree of diversity was noted for the second segment, in particular within the lysogeny module, where insertions, deletions, and DNA replacement events are common.[37,66] These molecular events are apparently also the cause for derivation of strictly lytic phages from temperate ancestors.[75]

3. *Lactobacillus*

For lactobacilli, completed phage genome sequences are available for five different species (*Lb. delbrueckii* subsp. *lactis, Lb. gasseri, Lb. casei, Lb. plantarum, Lb. johnsonii*) (Table 1). Phages from different *Lactobacillus* species do not share significant nucleotide sequence similarity. The genome of the *pac*-type *Lb. delbrueckii* subsp. *lactis* phage LL-H is closely related to the temperate *pac*-type *Lb. delbrueckii* subsp. *bulgaricus* phage mv4[22,23,76] and to phage 1b539.[77] The lytic *Lb. delbrueckii* subsp. *lactis* phage JCL1032 is unique since it shares DNA homology with lytic B1 Siphoviridae phages of the same species, although this phage has a prolate-headed morphotype of the B3 Siphoviridae.[77,78] The genome of the temperate *pac*-type *Lb. plantarum* phage ϕg1 is larger than the *Lb. delbrueckii* phage genomes (42,259 bp) and shares with these phages amino acid similarity within the structural proteins.[70] Determination of the complete genome sequence of *Lb. johnsonii* La1 revealed that the genome contains three uninducible prophages, Lj965 (39 kb), Lj928 (39 kb), and Lj771 (42 kb).[72] Phage Lj965 showed amino acid homology to proteins of *S. thermophilus* phage Sfi11 within the DNA packaging, head and tail morphogenesis modules. Phage Lj928 resembles *Lc. lactis* S114 prophage DNA within the DNA replication and packaging proteins. Furthermore, prophage Lj771 is related to the *Lb. gasseri* phage ϕadh within the tail fiber and lysis genes.

Phage ϕadh is a temperate *cos*-site phage which shares protein sequence similarity with the *cos*-site *S. thermophilus* phages within the DNA packaging, head and tail morphogenesis modules.[71,72] The temperate *Lb. casei cos*-type phage A2 possesses a few proteins within the structural gene module related to phage ϕadh.[68] The phage A2 DNA replication proteins revealed similarity with corresponding proteins of *S. thermophilus* phages (phages O1205 and Sfi21, respectively) and with *Lb. gasseri* phage ϕadh.[68]

4. *Comparative* Genomics

Comparative genomics has identified related phages in various species of lactic acid bacteria as well as in many nondairy species.[40,56,57] When the Sfi21-like and the Sfi11-like *S. thermophilus* phages were used as a central reference point, step-wise graded relatedness was observed on four different levels, ranging from complete DNA similarity to partial DNA similarity, protein similarity, and functional gene map similarity. Sfi21-like phages of *S. thermophilus* were very similar on the DNA level (level 1), while Sfi21-like phages from *S. thermophilus* and *Lc. lactis* (phage BK5-T) shared limited DNA similarity over the structural genes (level 2).[74] Sfi21-like *S. thermophilus* phages and *Lb. gasseri* phage ϕadh lacked DNA similarity but were linked by protein similarity over their structural genes (level 3). Finally, Sfi21-like *S. thermophilus* phages shared a similar organization of the functional gene map even with *E. coli* phages (level 4).[57] Sfi11-like *S. thermophilus* phages are similarly linked with a number of dairy and nondairy phages.[57] This hierarchy of relatedness was also observed for the various prophages in

one single *Lc. lactis* host (strain IL1403).[7] It has been proposed that Sfi21-like phages and Sfi11-like phages represent two new genera of Siphoviridae phages.[68]

D. Life Cycle

In the lytic cycle, the proliferation of phages in host cells results in cell lysis. This life cycle includes the following steps: adsorption, transcription, translation, DNA replication, DNA packaging, particle assembly, and release of phage progeny. The lysogenic life cycle of temperate phages differs in that these phages integrate as prophage into the host chromosome. Prophage DNA can excise from the chromosome either spontaneously or by induction. They may then propagate lytically or relysogenize again.

1. Common Steps

Phage Recognition/Adsorption/Infection. As typical viruses, phages must infect suitable (homologous) bacterial host cells for proliferation. Phage respect species borders but can easily propagate on different subspecies, as shown for the subspecies of *Lc. lactis* (i.e., subsp. *cremoris, lactis*, and *lactis* biovar. *diacetylactis*, respectively)[79–81] and for two subspecies of *Lb. delbrueckii* (i.e., subsp. *bulgaricus* and *lactis*).[22] Initially, phages must adsorb to the host cell. Frequently, adsorption occurs first in a reversible step, followed by an irreversible step.[82–84] For lactococci it has been shown by electron microscopy that two different types of phage attachment may occur (i.e., uniform adsorption of many phage on the whole cell surface versus unique adsorption of few phage in clumps to a limited number of receptor sites on the cell surface), reflecting the presence of different phage-specific receptors on the cell surface.[85] The latter type of adsorption is illustrated in Fig. 4.

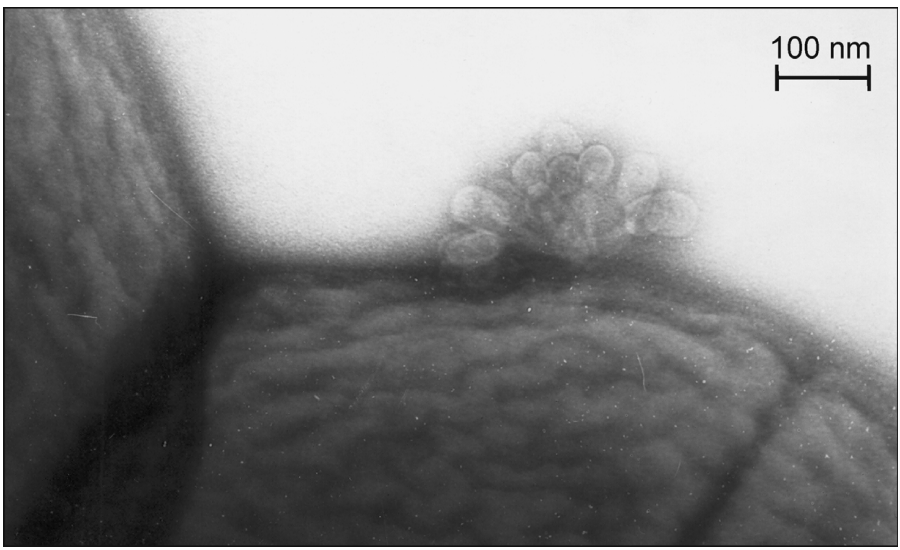

Figure 4 Site-specific adsorption of prolate-headed lactococcal phages on the surface of a *Lactococcus lactis* cell (transmission electron micrograph).

Carbohydrate components in the cell wall of *Lc. lactis, S. thermophilus*, and *Lb. casei* have been identified as the initial phage receptors. Rhamnose is essential, but further carbohydrate moities in the vicinity of the adsorption sites (i.e., galactose, glucose, or their acetylated forms) are also involved.[83,84,86–95] At least three different types of phage receptors have recently been proposed for *Lb. delbrueckii*,[96] two of which are specific for isometric-headed, and one being specific for prolate-headed phages.

Lb. helveticus strains were shown to be covered by a proteinaceous S-layer.[97] The central part of different S-layers was identified to be involved in playing a key role in phage adsorption.

The subsequently irreversible step of phage adsorption has been studied in detail for the prolate-headed lactococcal phage. Following the first reversible binding of phage c2 to the cell wall of *Lc. lactis* C2, phages adsorb irreversibly to a chromosomally encoded protein embedded in the cell membrane (designated phage infection protein, or PIP).[98] Only prolate-headed lactococcal phages but not phages of other species require a PIP protein for adsorption.[99] A likely transmembrane protein required for phage DNA injection has been identified in *S. thermophilus* by insertional mutagenesis.[100] The injection process of the phage PL-1 genome into *Lb. casei* ATCC 27092 cells requires cell energy from intact cells.[82,101] It has also been suggested that this process is dependent on the protein synthesis machinery of the bacterial host.[102]

Ca^{2+} or Mg^{2+} is indispensable for many phages infecting lactic acid bacteria (e.g., Refs.[103,104]), although some can also proliferate in the absence of these divalent cations (e.g., Ref.[105]). Ca^{2+} may not be necessary for the first phage-adsorption steps of various LAB phages but may be required for the latter processes of phage proliferation process.[86,106,107] Calcium-binding domains were found in the putative tape measure proteins of the prolate-headed lactococcal phages.[54,55] For phage LL-H infecting *Lb. delbrueckii* subsp. *lactis*, it has been proposed that the cations may function as counterions during the translocation of the phage DNA across the cell membrane.[108] A receptor-operated Ca^{2+}-channel system was discussed to be required for the transport of the DNA from *Lb. casei* phage PL-1 through the cell membrane of the host cells.[109]

In-depth phage genomics has also increased our knowledge about the phage structural proteins involved in host adsorption and recognition, and it has been suggested that a complex multiple-component system is possibly involved in host-range determination in dairy phages. These phage proteins are classified in four distinct families, exhibit a multidomain structure, and contain variable regions.[59] They have been identified in *S. thermophilus, Lc. lactis*, and *Lactobacillus* phages. Receptor-binding proteins of *S. thermophilus* and *Lc. lactis* phages (BK5-T, bIL286, bIL309) are putative tail fiber proteins with collagen-type motifs flanking a variable region.[7,66,110] This region has been described as a hot spot of recombination and is prone to deletion events.[64,110,111] A variable region within the C-terminal parts of these proteins was shown to be involved in host range specificity, as confirmed experimentally for phages of *S. thermophilus* and for prolate-headed lactococcal phages by the generation of chimeric phage mutants with altered host ranges.[112,113] It was shown by mutational analysis that a baseplate protein of the lactococcal phage TP901-1 was required for phage adsorption and for base plate assembly.[114] Similarly, it has been found that for lactococcal phages *orf18* in sk1 and *orf20* in bIL170 are involved in recognition of the bacterial host.[115] Host range mutants of *Lb. delbrueckii* phages LL-H and JCL1032, which were able to propagate on adsorption-blocking host mutants, revealed a distinct nucleotide change in the C-terminal part of the putative adsorption proteins.[96]

Transcription and Transcription Regulation. Phage DNA is injected as linear DNA into the host cell, and circularization occurs immediately by homologous recombination either of single-stranded *cos* ends or of the terminally redundant DNA of *pac*-type phages. Establishment of double *cos* sites was detected 1 minute after infection of host cells with the lactococcal phage ϕLC3.[116]

For dairy phages, two or three temporal classes of transcripts (early and late vs. early, middle, and late) have been reported. Only early and late but no middle transcripts have been reported for the virulent prolate-headed lactococcal phage c2 and for the virulent *Lb. delbrueckii* phage LL-H. In phage c2, transcription of the early and late genes occurs divergently.[117] Within the first 2 minutes of infection, a set of overlapping early transcripts was synthesized covering one third of the c2 genome. Early transcription was driven by very strong and simultaneously active early promoters by the host polymerase. Late overlapping c2-transcripts appearing 4–6 minutes after infection, initiated from a single late promoter, requiring a transcription activator. Early transcripts of the LL-H genes covering approximately 17% of the genome occur up to 20 minutes after infection, while late transcripts are detectable from 40 minutes postinfection until lysis. During the transcriptional gap between 20 and 40 minutes, LL-H DNA replication is started.[23]

Three classes of transcripts were detected during the lytic infection cycle of lactococcal phages sk1[118] and TP901-1[119] and of *S. thermophilus* phage Sfi21.[120] During phage sk1 infection, early transcription takes place 2–5 minutes after infection in opposite direction from the middle and late genes, which are transcribed 5–10 and 15 minutes postinfection. Evidence for phage-encoded transcriptional activators essential for the activity of middle- and/or late-inducible promoter has been reported in lactococcal P335-like phages (ϕ31,[121] TP901-1[122]) and in 936-like phages (sk1,[58] bIL41[123]) and also in *S. thermophilus* phage Sfi21.[120] Transcriptional starts in temperate phages can vary significantly: early transcription of TP901-1 started within the genetic switch region,[119] while early Sfi21 transcripts covered four different genome regions (12 genes between the DNA replication genes and the *cos* site, a single gene close to the *cos* site, a superinfection immunity gene in the lysogeny module, four genes between lysis and integrase genes).[120]

Transcription of prophage genes in the lysogenic host has been studied for lactococcal temperate phage BK5-T[124] and the streptococcal phages Sfi21 and O1205, repectively.[125] In all cases, only a few prophage genes are transcriptionally active. In the BK5-T prophage, transcription occurs from the *cI*-repressor gene, a superinfection exclusion gene, the integrase gene, and from an unidentified ORF located between the lysin gene and the *att* site. In the Sfi21 and O1205 prophages, the *cI* gene and the next two genes (including a superinfection immunity gene) further downstream are active, as are also a group of genes between the lysin gene and the *att* site.

DNA Replication. Replication of phage DNA is a critical step in the phage life cycle. DNA synthesis in the host cells starts 6 minute after infection by phage c6A[126] and results in high molecular weight progeny phage DNA (i.e., concatemeric forms), which is processed later in mature forms (linear single-length units).

DNA replication modules of lactococcal P335-like phages are organized in various ways. They contain either a single-stranded binding protein and a replisome organizer (replication initiator protein) (bIL285,[7] BK5-T,[60] TP901-1,[127] Tuc2009,[62] ul36,[8]),

a *DnaC* analog and a replisome organizer (r1t,[61] bIL309[7]) or occasionally all three genes (bIL286[7]). *Orf17* of phage Tuc2009 was discussed to determine a replication protein (helicase loader).[128] In general, the replisome organizers contain several direct repeats functional as origin of replications, confirmed experimentally for phage TP901-1.[127] The phage r1t DnaA analogue (Pro11 encoded by *orf11*) binds specifically to its own coding sequence spanning 47 bp with 6 bp short direct repeats.[129]

Genes for DNA polymerases were identified in c2- and 936-like lactococcal phages.[55,58] The origins of replication of phages c2 and sk1 were shown to function as a plasmid origin of replication in the absence of phage coinfection.[58,130] Analysis of c2-derived replication intermediates led to the conclusion that phage c2 replicates via a theta mechanism.[131] Phage ϕ31, a P335-phage with a strictly lytic lifestyle, has a replication module and an origin of replication closely related to *S. thermophilus* phages.[132]

The majority of *S. thermophilus* phages contain a highly conserved DNA replication module with three proteins all showing NTP-binding motifs. In phage Sfi21, two of these proteins revealed homology to putative DEAH box helicases and to plasmid-encoded primases. The Sfi21 origin of replication downstream of the primase gene shared similarity with the minus origin of replication of the cryptic *S. thermophilus* plasmid pST1.[133] Only a few *S. thermophilus* phages [7201,[134] TP-J34 (H. Neve and K. J. Heller, unpublished results)] possess a unique replication module with high similarity to protein analogues from phages of pathogenic streptococci (replication initiator protein A, DnaC, Erf-like protein, single-stranded binding proteins). Two functional origins of replication are present in the phage replication module of phage ϕ7201.[134] The replication modules of the lactobacilli phages ϕadh, A2, and ϕg1e were similar to the first-mentioned *S. thermophilus* type consisting of determinants for a NTP-binding protein, a helicase, and a primase.[70,71,135] In LL-H, the origin of replication was unusually located within a long noncoding region of the structural genes.[136]

Two types of genes specifying enzymes required for resolving Holliday junction intermediates are present in the replication module of a number of lactococcal phages. RusA-like proteins are known from P335-like phages (r1t,[61] ul36,[8] TP-901-1,[63] ϕ31.1,[10] bIL285[7]), while RuvC-like proteins were found in the 936-like phages bIL66[137] and bIL170.[59] For phage r1t it was shown that RusA binds to and cleaves specifically Holliday junction substrates. It is suggested that RusA cleaves branched phage DNA before packaging.[138]

All lactococcal P335 phages also contain a dUTPase determinant at the end of the replication module. These genes are the only conserved genetic elements in this heterogeneous phage group.[8] Activity of these enzymes is required to reduce the dUTP : dTTP ratio during phage genome replication. These determinants have not been found in 936- and c2-like lactococcal phages.

DNA Packaging. Terminases are required for phage DNA packaging, in that they mediate the ATP-dependent specific interaction between the phage prohead and its DNA. Two types of phage DNA packaging of individual progeny phage DNA molecules from a concatemeric precursor into the viral proheads are known: for *pac*-type phages, only the first cutting occurs at a *pac* site and subsequent "headful" quantities of DNAs are packaged exceeding one genome unit, giving rise to a heterogeneous population of terminally redundant and circularly permuted DNA molecules. Alternatively, in *cos*-type phages, cutting and subsequent packaging of individual progeny phage DNA molecules from the

concatemer occurs precisely at distinct *cos* sites, giving rise to a family of phage genomes with complementary protruding 3′ ends (cohesive ends). Cohesive ends of *cos*-site dairy phages are composed of single-stranded DNA with 3′ overhangs. The length of the *cos* ends varies from 9 nucleotides (*Lc. lactis* phage c2[139]) to 15 nucleotides (*S. thermophilus* phage Sfi21[140]). A different genome structure has been reported for the lactococcal Podoviridae phage asccϕ28 genome, which is linear with terminal proteins linked covalently to its termini.[141] Terminases are usually composed of both a small and a large subunit. *Cos* sites are located either upstream (*Lc. lactis* phage c2,[142] *S. thermophilus* phage Sfi21[140]) or downstream (*Lb. casei* phage A2[143]) of the small terminase determinant or were located within this gene (*Lc. lactis* phage ϕLC3[144]). Similarly, *pac* sites either precede the gene for the small terminase subunits (*Lc. lactis* phage TP901-1[63]) or are located within the gene (*S. thermophilus* phage O1205[65]).

Phage Assembly. Posttranslational processing of the structural proteins may be an important control mechanism for phage assembly. In *cos*-type dairy phages, maturation of the major head protein requires cleavage of the N-terminal sequence of major head protein revealing a distinct coiled-coil prediction by a ClpP-class protease (*S. thermophilus* phage Sfi21,[140] *Lb. gasseri* phage ϕadh[71]). The cleaved peptide may function as the scaffolding protein required for stabilization of the pro-head components. Other phage structural proteins are also processed proteolytically. A common cleavage sequence (Pho-Pho-Arg$\downarrow$) has been described for the processing sites within the major head, portal, and tape measure protein of the *Lb. casei* phage A2.[17] Proteolytic processing of the major head protein does not occur in *pac*-type dairy phages, and a separate scaffold protein gene was identified upstream of the major head gene of these phages (*Lc. lactis* phage ul36;[8] TP901-1,[63] O1205,[65] LL-H[23]). The major head protein of phage ul36 is unique and is closely related to *S. pneumoniae* phage MM1.[8]

Detailed tail structures of the *Lc. lactis* phage TP901-1 were identified by immunoelectron microscopy (i.e., neck passage structure, major tail structural protein, base plate protein[145]). By mutational analysis it was shown that the longest gene within the tail assembly module specifies the phage tape measure protein of phage TP901-1.[114] In-frame deletion or duplication of 29% of this ORF resulted in a shortening or lengthening of the phage tail. A branched baseplate and tail assembly pathway was proposed for phage TP901-1.[114]

Lysis Cassette. Phage-mediated lysis of the host cells requires the concerted action of two phage-encoded proteins—a holin and a lysin. Holins are small transmembrane proteins which upon oligomerization form lesions in the cytoplasmic membrane. These pores allow access of the phage lysin to the bacterial cell wall. Within the lysis cassette (module) of dairy phages, the holin gene normally precedes the lysine gene, but a reverse order was found in the lysis cassette of *Oenococcus* phages.[146,147] *S. thermophilus* phages usually possess two holin genes preceding the lysis gene.[148]

Two distinct classes of lysins are known from dairy phages. Muramidase-like lysins are known for a number of phages of *Lactococcus* (e.g., phage ϕLC3[149]), of *Lactobacillus* (e.g., phage mv1[150]), and of *Oenoccoccus* (e.g., phage fOg44[147]). Amidase-type lysins are also encoded by numerous phages of *Lactococcus* (e.g., phage ϕUS3[151]), of *Lactobacillus* (e.g., phage PL-1[152]), and of *S. thermophilus*.[148] Phage lysins exhibit a two-domain structure with a C-terminal substrate-binding domain and

an N-terminal catalytic domain. Some lysins have been identified as natural chimerae (e.g., *Lc. lactis* phage bIL170 [59]). A chimeric Tuc2009 lysin was constructed by fusing its catalytic N-terminal half to the C-terminal domain of the pneumococcal LytA amidase.[153] The lysis genes of the *Lactobacillus* phages mv1, ϕadh, and ϕg1e were able to complement phage λ S (holin) or R (lysin) mutants.[150,154,155] Traditionally, phage lysins are addressed as "endolysins." However, the N-terminal regions of the *Lb. plantarum* phage ϕg1e[156] and of the *Oenococcus* phage fOg44 lysin[157] were shown to be posttranslationally processed as a signal peptide (lysis from without).

According to their number of transmembrane domains, holins are classified into distinct classes: class I holins possess three transmembrane domains (e.g., *Lb. casei* phage PL-1[152]), while two transmembrane domains are present in class II holins (e.g., *Lb. plantarum* phage ϕg1e[158]). Some phage holins reveal characteristic dual start motifs (e.g., *L. lactis* phage c2[55]). The *Oenococcus* phage fOg44 holin is unique revealing four transmembrane domains.[147]

Release of phage particles completes the lytic phage infection cycle. For lactococcal phage, burst sizes ranging from <10[12,159,160] to 400[8] and latent periods from 10 to 140 minutes have been documented (reviewed in Refs.[161,162]).

2. Lysogenic Cycle

Phage Decision/Genetic Switch. While infection of a host cell with virulent phages will usually result in host cell lysis, temperate phages have to decide early in the infection cycle to enter either the lytic or the lysogenic pathway. It is estimated that for the *S. thermophilus* phage Sfi21 and the lactococcal phage ϕLC3, approximately 1 out of 1000 infected cells follow the lysogenic instead of the lytic pathway.[116,125] This decision is made by the genetic switch of temperate phages. Lysis of a lysogenic lactococcal cell and release of temperate phages is illustrated in Fig. 5.

A genetic switch closely related to the phage λ model was identified in the *Lb. casei* phage A2. CI- and Cro-like regulatory proteins (repressors) are divergently transcribed from their corresponding promoters P_L and P_R, respectively. CI represses the lytic phage cycle and promotes the maintenance of the lysogenic cycle. The repressors bind to three 20 bp operator sites (O_1, O_2, and O_3) within the intergenic region between the *cI* and *cro* genes. CI binds cooperatively to O_1 and O_2 (overlapping P_R) and at higher concentrations also to O_3 (overlapping P_L), while Cro exhibits a reverse binding affinity.[163,164]

The genetic switches of other dairy phages are organized differently. The number of operator sites may vary from two (*S. thermophilus* phage Sfi21[165]) up to seven (*Lb. plantarum* phage ϕg1e[166]). Operator sites not only were found within the intergenic switch region overlapping the *cI* and *cro* promoters, but were located either within (lactococcal phage r1t[167]) or at the 3′-end of the *cro* topologues (lactococcal phages TP901-1[168] and ϕ31;[169] *S. thermophilus* phage Sfi21[165]). Differential binding of the CI- and Cro-like repressors to the seven operator sites was shown for phage ϕg1e.[170,171] The CI repressor of phage *Lb. gasseri* ϕadh represses the transcription from the *cro* and *cI* promoters 20- and 5-fold, respectively.[172]

On the lactococcal temperate phage BK5-T genome, the divergently orientated *cI* and *cro* genes are separated by an *ORF* of unknown function which is located several ORFs further downstream of the switch region in other lactococcal phages like TP901-1.[60] The *cro* topologues of lactococcal phages TP901-1 and ϕLC3 and of the *S. thermophilus* phage Sfi21 do not bind to the genetic switch region.[165,173,174] For these phages,

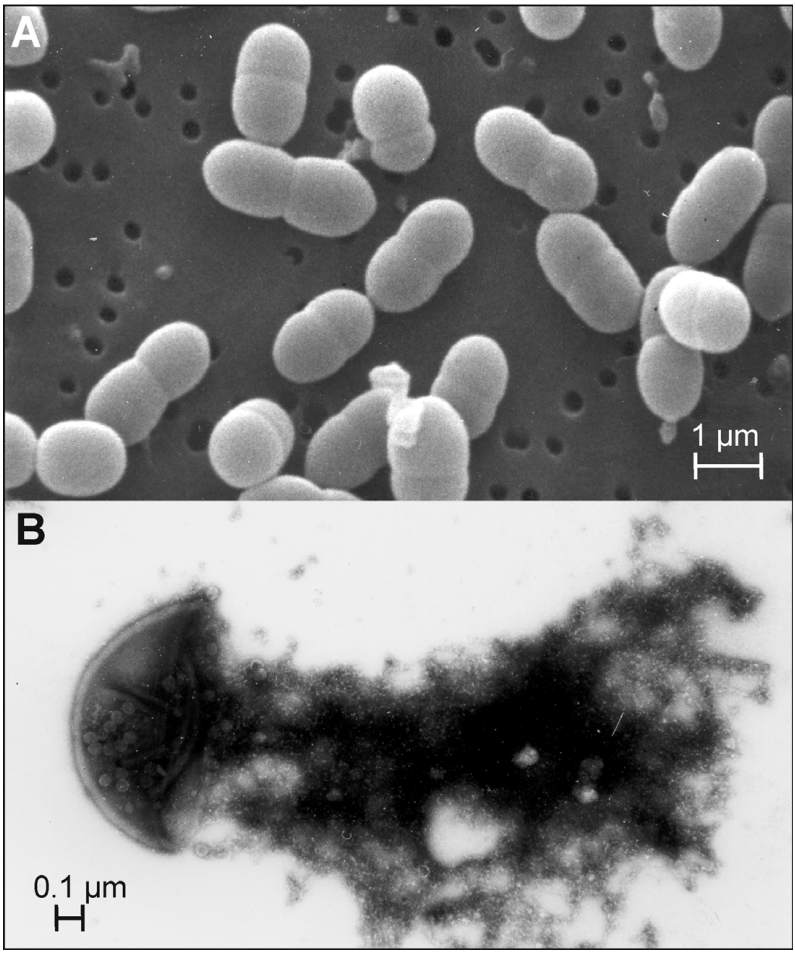

Figure 5 Lysis of a lysogenic lactococcal cell and release of temperate phages (transmission electron micrograph B) after treatment of the *Lactococcus lactis* culture (scanning electron micrograph A) with mitomycin C.

a protein-protein interaction between both repressors prevents the binding of the CI repressor to the genetic switch.

In many dairy phages, a gene for a putative antirepressor protein is frequently located downstream of the *cro* homologue, but their function has not been confirmed experimentally.[175] Deletions in the antirepressor gene of the *S. thermophilus* phage Sfi21 did not affect the lytic growth of the phage mutants but resulted in a delayed establishment of the lysogenic state.[165] A putatively defective antirepressor was also reported for *Lb. casei* phage A2.[17]

Integration and Excision of Prophages. Temperate phages require site-specific recombination proteins for integration into and excision from the host chromosome. All except one dairy temperate phage use tyrosine integrases of the Int family of site-specific recombinases for prophage integration, e.g., *Lc. lactis* phage ϕLC3,[176] *S. thermophilus* phage

Sfi21,[34] *Lb. delbrueckii* subsp. *bulgaricins* phage mv4,[177] and *Oenococcus* phage fOg44.[147]

The integrase of the lactococcal phage TP901-1 is unique and belongs to the family of extended resolvases.[178] The minimum sizes of *attB* and *attP* allowing efficient recombination were 43 and 56 bp, respectively.[179] The TP901-1 integrase also allows a low level of prophage excision, but full excision activity requires an excisionase coded by the third gene in the early lytic operon (ORF7) located 6 ORFs upstream of the TP901 integrase gene.[180] This constellation of *int* and *xis* is highly unusual. Excisionases are so far unknown for the majority of dairy temperate phages. A unique and very acid excisionase was identified directly upstream of the *Lb. plantarum* phage ϕg1e integrase gene.[181] Besides this *xis-int-attP* genetic organization, an *xis-attP-int* constellation has been reported for the *Oenococcus* phage fOg44.[147]

Lactococcal and *S. thermophilus* integrases show the closest relatedness.[182] The lactococcal phage ul36 integrase is more closely related to *S. pyogenes* phage integrases than to other *Lc. lactis* phage integrases.[8] Integration is mediated between short homologous sequences of the phage genome (*attP*) and the host chromosome (*attB*). *AttP* is generally located downstream of the integrase gene but was also found overlapping the 3′ end of *int* as shown for the *S. thermophilus* phages Sfi21 and TP-J34.[37,183] The length of the common core sequence mediating the DNA strand exchange reaction differs significantly, ranging in size from 5 to 9 bp (*Lc. lactis* phages ϕLC3[176] and TP901-1[184]) up to 40 bp (*S. thermophilus* phage Sfi21,[183] *Lb. casei* phage ϕFSW[16]). Multiple integration sites of prophages into the host chromosomes are known, including 3′ ends of tRNA genes ($tRNA^{Ser}$,[177] $tRNA^{Leu}$,[185,186] $tRNA^{Arg}$[37,183]). Integration sites of dairy temperate phages were also identified in ORFs of known or unknown functions or in intergenic regions.[7,16,180]

Endonucleases and Introns. The *Lc. lactis* phage bIL170 possesses four genes for homing endonucleases of the HNH family[59] as free-standing ORFs between genes. Homing endonucleases are also known to be encoded within self-splicing introns at different genome positions in the *Lc. lactis* phage r1t,[61] in the *Lb. delbrueckii* subsp. *lactis* phage LL-H,[187] and in the *Lb. casei* phage A2.[17] Introns are particularly widespread in *S. thermophilus* phages; nearly one half of these phages contained a group IA2 intron interrupting the lysin gene.[188]

3. Pseudolysogeny (Phage Carrier State)

Incidences of true lysogeny must not be confused with pseudolysogeny. As indicated by the term "phage carrier state," pseudolysogeny results from a permanent infection of a (usually undefined mixed strain) starter culture by a virulent phage. These phages persist in low titers in the culture due to their propagation in a limited number of phage-sensitive cells in the culture.[189–191]

E. Origin of Phage and Phage Evolution

Many food fermentations are performed in a nonsterile environment. In dairy plants, phages will survive the heat treatment of milk used for cheese production.[192] Raw milk is also used for the production of a variety of traditional cheeses.[193] The discussion regarding the main routes of phage contamination in the dairy plant was controversial, but a few sources have been clearly identified (i.e., infection by lysogenic starter cultures and phages propagating on raw milk bacteria).

It was shown for the temperate *Lb. casei* phage ϕFSW that it could mutate to a virulent phage form (ϕFSV) during the production of Yakult.[194] Replacing the lysogenic wild-type strain by a prophage-cured derivative efficiently eliminated this phage-infection route. This transition from a temperate to a virulent phage was due to the acquisition of a 1.3 kb insertion element (ISL*1*) by the virulent phage, which allowed this phage to infect the original lysogenic host.[195]

Deletions occurring within the lysogeny modules of temperate phages affecting the genetic switch region of the site-specific integration system result in the emergence of new lytic phages: lytic derivatives of the temperate *S. thermophilus* phage ϕSfi21 and lactococcal temperate phage BK5-T were unable to reenter the lysogenic cycle due to spontaneous site-specific deletions within their lysogeny module covering part of the integrase gene and the *attP* site.[75,110] Lytic *S. thermophilus* phages showed a replacement module derived from lysogeny modules by insertion/deletion and DNA rearrangement processes.[66] The strictly lytic P335-type phage ϕ31 also lacks a functional integrase gene and *attP* region[132] but still contains a genetic switch with a dysfuntional *cI* gene.[169] A truncated integrase gene was also detected in the genome of the virulent *Lb. delbrueckii* subsp. *lactis* phage LL-H,[196] which otherwise is closely related to the temperate *Lb. delbrueckii* subsp. *lactis* phage mv4. A clear-plaque mutant of the lactococcal temperate phage ϕLC3 was shown to have suffered an amber mutation in the repressor gene.[174]

Raw milk is considered to be an important source of phages that propagate at low levels on phage-sensitive nonstarter lactic acid bacteria.[81,197,198] Hence it has been possible to isolate lactococcal phages directly from raw milk samples.[79] For a mozzarella cheese factory it was shown that the genetic diversity of *S. thermophilus* phages was not created in the factory but apparently was already present in the natural environment (i.e., in the raw-milk).[199]

Lactococcal P335-like phages have either a lytic or a lysogenic life style and share all DNA homology. Hence, lytic P335-like phages can exchange DNA with prophages and prophage remnants present on the bacterial host chromosome. It is well known that this phage group is particularly "promiscous," allowing the incorporation of various amounts of prophage or temperate phage DNA into the genomes of lytic phages.[200] Recombinant lytic P335-like phages with altered genome organizations have been isolated that were able to overcome host defense mechanisms.[10,201,202] Acquisition of a new DNA replication module and a new origin of replication was documented for the P335-like phages ϕ31 and ul36. Recombination possibilities could be significantly reduced by insertional disruption of the prophage DNA involved in the DNA exchanges in the chromosomes.[10] P335-phages could also acquire new DNA from a plasmid source.[203]

F. Monitoring

Traditional methods for monitoring phages in the dairy field (activity tests, plaque assays) are reliable but have the disadvantage of being time-consuming and require the availability of suitable indicator strains. Methods to detect phage directly on the DNA level in whey samples proved successful using the dot blot technique (limit of detection: 10^5 plaque-forming units (PFU) per dot;[204]) or by PCR approaches (limit of detection: 10^3 PFU/mL whey).[31] A multiplex PCR strategy was designed to detect in a single reaction the presence of the three most prominent lactococcal c2, P335, and 936 phages.[205] Alternatively, phage-specific antisera facilitated the rapid, direct serological detection

of phage in milk or whey samples within several hours by enzyme-linked immunosorbent assays (ELISA). Polyclonal antisera raised against native lactococcal c2- and 936-like phages were used originally.[206,207] Monoclonal antibodies were applied later specific for the denatured major capsid protein of P335 phages[208] or raised against native c2-like phages.[209] The limit of detection of these ELISA-based assays was low (10^7 PFU/mL).

III. PHAGE DEFENSE MECHANISMS

A. Naturally Occurring Antiphage Mechanisms

Lactic acid bacteria have been used in food fermentations for thousands of years and consequently, have been exposed to phages continuously. The strong dynamics of this intimate phage/host relationship has inevitably resulted in the manifestation of a panoply of different naturally occurring phage insensitivity mechanisms which have been studied in detail especially within lactococci. With respect to the phage life cycle described above, phage insensitivity can act at four different subsequent steps: adsorption inhibition, DNA penetration blocking, restriction/modification and finally abortive inhibition. In lactococci, many of these systems are linked with plasmids. Similar systems, some of which are not plasmid-encoded, have also been detected in *S. thermophilus*. Failure of lactococcal phages to adsorb to a *Lc. lactis* cell is illustrated in Fig. 6.

1. Adsorption Inhibition

Attachment of a phage to the cell surface is a very specific process, dependent on phage specificity, accessibility of bacterial receptor, physiochemical properties of the cell

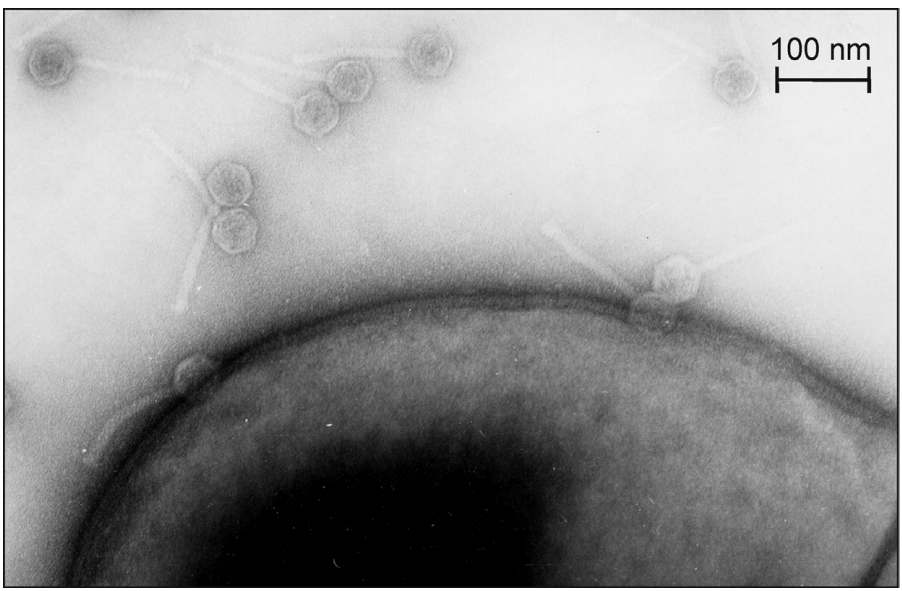

Figure 6 Transmission electron micrograph of a phage-resistant *Lactococcus lactis* cell that does not allow adsorption of lactococcal isometric-headed phages.

envelope, and the electrical potential across the cytoplasmic membrane.[210] In gram-positive bacteria a thick multilayered peptidoglycan is considered to be responsible for the mechanical integrity and is located on the outside of the cell.[211] In this layer different components such as polysaccharides, lipoteichoic acid, and proteins stick out. Thus, Boonaert and Rouxhet[212] found that the surface of *Lc. lactis* was covered predominantly by polysaccharides and proteins in the ratio 2 : 1, while Lortal et al.[213] found that an S-layer composed of a protein covered the surface of *Lb. helveticus* strains. Change in the amino acid sequence can affect phage adsorption.[97] The phages first recognize a polysaccharide before they attach to a protein located in the plasma membrane. The polysaccharides are often composed of rhamnose, glucose, galactose, and glucosamine[89,91,214,215] and are covalently linked to the peptidoglycans.[87] The composition/structure of the polysaccharides most likely varies between different strains. The first step may be reversible, while the last step involving injection of phage DNA is irreversible.[84] Failure of a phage to adsorb to a host cell (see Fig. 6) may be due to either the lack of appropriate polysaccharides on the cell surface or to a physical masking of the receptor polysaccharide or protein.

The hypothesis that phages recognize an appropriate polysaccharide or that saccharides are involved in recognition is supported by several observations.[87,89,216] Gopal and Reilly[87] found in a spontaneous phage-resistant variant unable to adsorb small isometric-headed phages that the amount of galactose and glucosamine was reduced 15-fold and 3-fold, respectively, compared to the parental strain. Tuncer and Akcelik[216] found a galactose-containing receptor site for four phages.

The masking may be exhibited by production of either lipoteichoic acid, a protein, or exopolysaccharides. Sijtsma et al.[210] identified the shielding components encoded by plasmid pSK112 from *Lc. lactis* subsp. *cremoris* SK110 as galactosyl-containing lipoteichoic acid, while Tuncer and Akcelik[216] in *Lc. lactis* subsp. *lactis* MPL56 found that a plasmid-encoded protein of 55.4 kDa blocked the receptor sites.

Polysaccharides may either be excreted into the environment as exopolysaccharides (EPS) or form capsular polysaccharides (CPS). It has been discovered that production of EPS in some cases blocks adsorption,[217] but not always.[218] CPS seems to provide bacteria with a higher degree of protection than EPS (J. Josephsen, unpublished results). Table 2 shows plasmid-encoded mechanisms identified as blocking adsorption in *Lactococcus.*

2. Blocking of DNA Penetration

In *Lc. lactis* C2, reversible adsorption of phage ϕc2 to the lactococcal cell wall is followed by irreversible adsorption to a phage infection protein (PIP) located in the cell membrane.[227] A phage-resistant mutant of strain C2 which could still adsorb the phage was shown to be defective in the phage infection protein. Hence, phage c2 could be inactivated by cell membranes isolated from strain C2 but not by a purified membrane fraction from the PIP-deficient mutant.[84] PIP is encoded by a chromosomal gene of strain C2. Phage resistance due to blocking of phage DNA penetration can also be plasmid-encoded as shown for pNP40 from *Lc. lactis* subsp. *lactis* DRC3.[228] Inhibition of PL-1 phage genome injection has also been suggested as the underlying mechanism for phage insensitivity of *Lb. casei*.[215] A membrane protein involved in the DNA injection process has also been identified in *S. thermophilus* by insertional mutagenesis.[100]

Table 2 Plasmids Identified as Blocking Adsorption in *Lactococcus*

Plasmid	Size (kb)	Host	Blocking component	Ref.
pSK112	54	LC SK110	Lipoteichoic acid	210
NN	28.5	LL MPL56	55.4 kDa protein	216
p2520L	37.5	LL P25	Surface antigen	219
pKC50	80	LL 57150	Surface antigen	220
pCI658	58	LC HO2	Exopolysaccharide	221,222
NN	16.5	LC MA39	Exopolysaccharide	223
pCI528	46	LC UC503	Exopolysaccharide	217
pAH90	26.5	LLD DPC721	Undetermined	224
pME0030	48	LL ME2	Undetermined	225
p1149-1	13	LC 11/49	Undetermined	226

LL, *Lactococcus lactis* subsp. *lactis*; LC, *Lactococcus lactis* subsp. *cremoris*; LLD, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*; NN, no name assigned to plasmid.
Compiled with the assistance of Aidan Coffey, Cork Institute of Technology, Ireland.

3. Restriction/Modification Systems

Plasmid-encoded restriction/modification (R/M) systems are widespread in lactic acid bacteria, particularly in lactococci. Many *Lactococcus* strains probably harbor more than one R/M system, as several strains have been observed to contain more than one R/M encoding plasmid([229–231]; J. Josephsen, unpublished results), indicating their importance in defending the strains against phages. An R/M system first recognizes a specific DNA sequence; then it either modifies DNA by a methyltransferase or cleaves DNA with a restriction endonuclease. Methylation is performed either on adenine or cytosine located within the recognition sequence by transfer of a methyl group from *S*-adenosyl-L-methionine. The bacterium methylates its own DNA, thereby protecting it against restriction. Cleavage by the restriction endonuclease will take place either within or nearby the recognition site or randomly. Therefore the R/M system can protect bacteria against foreign DNA such as phages by cleaving invading phage DNA. In general the R/M systems have a broad range of efficiency and can inactivate many different kinds of phages as they are not dependent on phage species or morphology. It only requires that the recognition sequence specific for the R/M system is present in the phage DNA and that this sequence has not been modified by methylation. The DNA of progeny phages that has escaped restriction by an R/M system will be methylated, and therefore these phages can circumvent that specific R/M system and reinfect the bacteria with a high efficiency. Therefore R/M systems alone are not very powerful; however, combined with other R/M systems or other kinds of defense mechanisms, they are more powerful. The efficiency with which they are able to restrict phages depends on the number of recognition sites in the phage DNA. As the number of recognition sites in viral DNA increases, the EOP decreases exponentially.[232]

R/M systems are grouped into four main types.[233] Until now mainly type II R/M systems have been characterized from *Lactococcu*s and *S. thermophilus.* R/M systems recognizing 5′-GATC-3′ as LlaAI,[234,235] LlaCDHI,[236] and LlaKRI[237] seem to be very widespread not only in *Lactococcus*, where we found LlaAI to be present in at least 12 out of 62 isolates from a mixed Cheddar starter culture,[234] but also in many

other genera of bacteria, both gram-negative and gram-positive. It has also been found in *S. thermophilus* strains both on a plasmid[238] as well as in the chromosome.[239]

However, most R/M systems, at least in *Lactococcus*, do not properly belong to type II.[234] A few type I,[224,240,241] one type III,[242] and some unusual R/M systems[231,243,244] have now been discovered. In *Lactococcus* (Accession numbers: AF228680, U90222, and AF034786) and *S. thermophilus* (Accession numbers: AJ315964, AF177167), all the known genes belonging to type I R/M systems encoding the HsdR are almost identical. The same is the case for the HsdM subunits, while the specifity subunits HsdS are different. Figure 7 shows the gene products and putative motifs of a type I RM system and the two unusual systems LlaGI[243] and LlaBII (J. Josephsen, unpublished). The LlaGI system is unusual, as it consists of only one open reading frame of 4683 or 4710 base pairs (depending on which start codon is used) and does not express type II endonuclease activity. Analyses of its predicted amino acid sequence revealed the presence of a catalytic motif and seven helicase-like motifs (DEAD-box motif) characteristic of type I and III endonucleases, followed by four conserved methylase motifs characteristic of adenine methylases. The presence of helicase motifs indicates that before cleavage it translocates DNA in an ATP-requiring process, resulting in random cleavages outside its recognition site. It was suggested to belong to a variant of type I systems due to the order of its motifs and because its methylase motifs belongs to class N_{12}, as do type I, while type III methylases belong to class D_{21}. Furthermore, the lack of a promoter near the start codon suggests

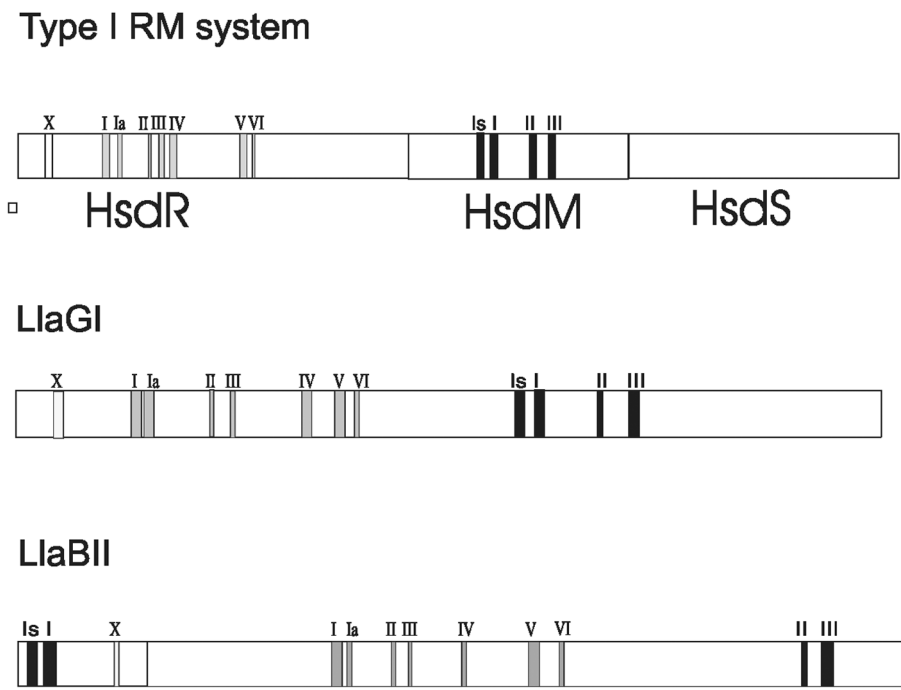

Figure 7 Schematic drawing of gene products with motifs indicated from three different restriction modification systems. The open boxes labeled X represent the catalytic motif, the grey boxes (I–VI) represent the DEAD-box motifs, and the black boxes the methylase motifs (CMI, CMIs, CMII, and CMIII). The drawing is not to scale.

that transcription starts from the promoter located upstream of *repB*. The same has been suggested for various lactococcal type I R/M systems.[245] In general, we have observed that plasmid-encoded R/M systems are very often located downstream of the replication region.

The LlaBII system is even more peculiar. It consists of two open reading frames, *llaAIIM*, coding for 230 amino acids, and *llaAIIRM*, encoding 1462 amino acids. It does not express type II endonuclease activity. Analysis of its amino acid sequences revealed the presence of two methylase motifs, CMIs and CMI, and maybe a catalytic motif in the small LlaAIIM subunit; seven helicase-like motifs followed by the methylase motifs CMII and CMIII typical for adenine methylases were identified in the large LlaAIIRM subunit (J. Josephsen, unpublished results). Normally all the methylase motifs are located in the same open reading frame. It is also here presumed that the LlaBII system translocates DNA in an ATP-dependent process before cleavage. Table 3 shows all the R/M systems in lactic acid bacteria presently published.

Besides complete R/M systems, it also seems that *Lactococcus* harbors plasmids that carry genes encoding only the HsdS subunit, alone or together with another HsdS subunit or other antiphage mechanisms([245,264,265]; J. Josephsen, unpublished results). These subunits can combine both with chromosome- or plasmid-encoded type I R/M systems and alter their specificities. It is also found that by homologous recombination they can generate chimeric HsdS subunits with new specificities.[266] Therefore, especially type I R/M systems seem to provide bacteria with flexible defense mechanisms that can be evolved to withstand the continuous development of new phages.

It is also noteworthy that in type II systems in lactic acid bacteria, some genes encoding the restriction endonuclease have an unusually high similarity to their isoschizomers identified in other genera, e.g., *Enterococcus*, *Streptococcus*, and *Neisseria*, as shown in Table 4. This indicates that lactic acid bacteria growing in milk have developed some degree of protection against phages by horizontal transfer of genes encoding R/M systems from other genera of bacteria.

4. Abortive Infection Mechanisms

When phages have adsorbed to a host cell and subsequently succeeded in injecting their genome into the cell interior without being fragmented by the activity of R/M systems, subsequent intracellular development (i.e., DNA replication, transcription, translation, DNA packaging, assembly of phage progeny) is the last crucial step for the host to combat the phage attack. However, since phage maturation in the host cell causes degradation of the host chromosome, death of a significant portion of the culture will occur. Hence, phage will remain entrapped in the host cell and will not be released into the environment. These phage insensitivity systems are thus designated abortive infection (Abi) systems and are easily detected either by a complete absence of phage-derived plaques or by a severe reduction in plaque size, decreased efficiency of plating (EOP) and burst size, and reduction in the efficiency at which centers of infection are formed (ECOI).

Abi systems are widespread in lactococci, and at least 21 different Abi systems have been identified (Table 5). Most of them are plasmid-encoded. Generally comparisons of Abi sequences indicate diverse origins, presumably due to action on many different steps in the phage life cycle; however, there are a few exceptions, such as AbiF, which is 26% identical to AbiD and 46% to AbiDI;[267] AbiA, found on two different plasmids (pTRK2030 and pCI829), is similar to AbiK. This suggests the presence of families of Abi

Table 3 Restriction/Modification Systems Characterized in *Lactococcus lactis* and *Streptococcus thermophilus*

Name	Host	Specificity[a]	Location	Ref.
Type I				
Lla1403I	LL IL1403	Undetermined	Chromosome	241
Lla42oI[b]	LC IL420	Undetermined	pIL2614	245
Lla82I	LL DPC220	Undetermined	pAH82	224
Lla90I	LL DPC721	Undetermined	pAH90	224
LldI	LL LD10-1	Undetermined	pND861	240
Sth135I	ST 135	Undetermined	pER35	246
SthSFiI	ST Sfi1	Undetermined	Chromosome	100
Type II				
LlaAI	LC W9	5′ ↓ GATC 3′	pFW094	234,235
LlaDCHI	LC DCH-4	5′ ↓ GATC 3′	pSRQ700	236,247
LlaKR2I	LL KR2	5′ ↓ GATC 3′	pKR223	237
Sth8I	ST 8	5′ GATC 3′	pSt08	Accession no. AJ239049
Sth368I	ST CNRZ368	5′ GATC 3′	Chromosome	239
LlaCI	LC W15	5′ A ↓ AGCTT 3′	pAW153	248,249
LlaBI	LC W56	5′ C ↓ TRYAG 3′	pJW563	234,235,250
LlaDI	LC W39		pJW394	Accession no. AJ292520
LlaG2I	LC	5′ G ↓ CTAGC 3′	Unspecified	B. Karska-Wysocki, Rebase
Lla497I	LL NCDO497	5′ CCW ↓ GG 3′	Unspecified	251
Sth117I	ST 117	5′ CC ↓ WGG 3′	Chromosome	252
SslI	ST T	5′ CCWGG 3′	Chromosome	253
Sth455I	ST CNRZ455	5′ CCWGG 3′	Chromosome	254
ScrFI	LC UC503	5′ CC ↓ NGG 3′	Chromosome	255–258
LlaMI	LCM19	5′ CCNGG 3′	Unspecified	259
LlaDII	LC W39	5′ GC ↓ NGC 3′	pHW393	260
Sth0I	ST 0	5′ GCNGC 3′	pSt0	238
Sth134I	ST 134	5′ C ↓ CGG 3′	Chromosome	261
Sth132I	ST 132	5′CCCG(N)4 3′, 3′ GGGC(N)8 5′	Chromosome	262
Type III				
LlaFI	LL 42-1	Undetermined	pND801	242
Nonclassical type				
LlaGI	LC W10	Undetermined	pEW104	243
LlaBIII	LC W56	Undetermined	pJW566	231 J. Josephsen, unpublished
LlaBII	LC W56	Undetermined	pJW565	231; Accession no. Y12736
LlaI	LL ME2	Undetermined	pTR2030	244,263

LC, *L. lactis* subsp. *cremoris*; LL, *L. lactis* subsp. *lactis*; ST, *Streptococcus thermophilus,* W = A or T; R = A or G; Y = C or T; N = ACG or T.

[a]The cleavage point, where known, is indicated by ↓.

[b]Designated Lla2614I in REBASE.NEB.COM

Source: Compiled with the assistance of Aidan Coffey, Cork Institute of Technology, Ireland.

Table 4 Comparison of Type II Restriction Endonucleases Isolated from *Lactococcus* with Their Nearest Isoschizomer[a]

Name of RM system	Name of isoschizomer	Origin of isoschizomer	Number of a.a. in REase/ isoschizomer	% Identity (in a.a. out of)	% Similarity (in a.a. out of)	Accession numbers: lactococcal REase/ isoschizomer
LlaBI	SfeI	*Enterococcus* (formerly *Streptococcus*) *faecalis*	299/293	97% (280/286)	97% (282/286)	X97263/AY151403
LlaDII	SthSt0IP	*Streptococcus thermophilus*	180/180	82% (148/179)	90% (164/179)	Y12707/AJ242480
	Bsp6I	*Bacillus*	180/174	42% (67/156)	64% (50/57)	Y12707/X81638
LlaDCHI/ LlaAI	Ssu4109IB	*Streptococcus suis*	304/301	73% (221/300)	83% (258/300)	NC_002798/ AB058945
LlaKR2I	Sau3AI	*Staphylococcus aureus*	496/489	33% (160/472)	51% (246/472)	AF051563/M32470
LlaDI	NmeSI	*Neisseria meningitidis*	252/199	33% (77/200)	55% (111/200)	AJ292520/ AF123569
LlaCI	EcoVIII	*Escherichia coli*	332/307	26% (29/108)	48% (53/108)	AJ002064/ AF158026
ScrFI	LlaMI	*Lactococcus lactis*	271/271	97% (263/271)	97% (265/271)	U89998/AF487827

Table 5 Abortive Infection Systems Characterized from *Lactococcus*

Name	Mechanism	Host	Phage species affected	Number of amino acids	Location	Ref.
AbiA	Early	LL ME2	936, P335, c2	628	pTR2030	281–285
		LL BA2			pCI829	286,287
AbiB	Late	LL IL416	936	250	Chromosome pCI642	123,288 222
AbiC	Late	LL ME2	936	344	pTN20	289
AbiD	Late	LL KR5	936, c2	366	pBF61	290
AbiD1	Late	LL IL964	936	351	pIL105	291–293
AbiE	Late	LL DRC3	936	286, 297	pNP40	277
AbiF	Early	LL DRC3 LL 4942	936, c2	341	pNP40, pAJ2074	277,294,295
AbiG	Late	LC 320	936, c2	249, 397	pCI750	279,296
AbiH	—	LL S94	936, c2	346	Chrom.	297
AbiI	Late	LL M138	936, c2	331	pND852	298
AbiJ	—	LL UK12922	936	282	pND859	299
AbiK	Early	LL W1	936, P335, c2	599	pSRQ800	270,275
AbiL	Late	LL LD10-1	936, c2	458, 297	pND861	300
AbiN	—	LC S114	936, c2	178	Chromosome	301
AbiO	—	LL S45-91-1	936, c2	540	pPF144	302
AbiP	Early	LC IL420	936	244	pIL2614	M. C. Chopin, personal communication (Accession no. U90222)
AbiQ	Late	LL W-37	936, c2	183	pSRQ900	247,274
AbiR	Early	LL KR2	936, c2	2 loci, (4 ORFs)	pKR223	J Josephsen unpublished (Accession no. Ad132009)
AbiS	—	LC W60	936	DNA structure	pAW601	268
AbiT	Late	LL W51	936, P335	127, 213	pED1	280
AbiU	Early	LL51-1	936, P335, c2	589, 341	pND001	273

mechanisms. All Abi systems are characterized by an unusually low G + C content of their genes (26–29%), which differs significantly from the 37% average determined for other known lactococcal genes. The AbiE, AbiG, AbiL, AbiR, AbiT, and AbiU phenotypes require two genes, while all the other Abi systems except AbiS only require a single gene.

AbiS involves noncoding sequences with high homology to the *cos* region of 936 phages and regions repeated several times, as shown in Fig. 8 (J. Josephsen, unpublished results).

Little is known about the modes of action of the Abi systems, and often they are only categorized according to their ability of acting early (before or on DNA replication) or late (after replication) in the lytic cycle of the phage development;[269] however, a few systems have been characterized in more details. Furthermore, it has been found that the mode of action for some Abi system like AbiK and AbiG is distinct for the different phage species.[270,271] This indicates that the proteins encoded by *abiK* and *abiGii* may be multifunctional with more than one active site, as suggested for proteins larger than 300 amino acids.[271,272] A drawback is that not all of the systems have been examined against the three main phage species. All systems affect 936 phages, 11 systems also affect c2 phages, while three systems affect all the three main species. AbiA, AbiG, and AbiK generally affect the 936 phages stronger than the c2 phages, while AbiU affects c2 more than 936 phages.[273] AbiQ is very effective against both 936 and c2 phages.[274] The AbiA, AbiK, AbiF, and AbiR systems act on the phage DNA replication or prior to this in the host cells.[275–277] AbiG, AbiU, and an undefined Abi system encoded by plasmid pBU1-8 from *Lc. lactis* subsp. *lactis* Bu2 inhibits or delays transcription of phage DNA.[226] AbiB was shown to prevent phage growth by promoting degradation of transcripts derived from phage bIL170 (936 species) infection of *Lc. lactis* subsp. *lactis* IL1403 starting 10–15 minutes after infection.[123] It has been suggested that an early product of the phage either induces the synthesis or stimulates the activity of an RNase in the $AbiB^+$ cells. Using monoclonal antibodies raised against the major capsid protein of phage u136 in an ELISA test system, it was shown that the production of this structural protein was reduced by 50% in lactococcal host cells harboring the AbiC system.[278] AbiE, AbiG, AbiQ, and AbiT also act at a late stage in phage development, since phage DNA replication is not inhibited.[274,277,279,280] In cells harboring AbiQ, the immature concatemeric form of phage DNA accumulated in the cells. This suggests that it may be defective and unable to be processed into mature phages or that genes involved in phage morphogenesis are affected by AbiQ.[274] It has been suggested that one of the late mRNAs or proteins activates the AbiT mechanism and causes premature cell death.[280]

Phage mutants capable of overcoming the abortive infection mechanism (i.e., Abi-resistant mutants) have been used to identify the Abi targets on the phage genome. By this approach, mutants from lactococcal phage bIL66 (936 species) capable of overcoming the

Figure 8 Schematic drawing of plasmid pAW601 encoding AbiS. The grey arrows show the location of the sequences with homology to the *cos* region of phage sk1. The open arrows show the location of identical sequences of 96–98 bp; the black arrows the locations of identical sequences of 147 bp. The arrow marked repB is an open reading frame with high homology to theta replication proteins.

AbiD1 phage resistance were isolated.[293] It has been suggested that the terminal part of Orf1 protein interacts with the AbiD1 gene product to decrease the amount of the gene product from the third *orf*, encoding an endonuclease homologous to *Escherichia coli* RuvC resolvase, below a critical level required for DNA maturation prior to packaging.[304]

In a similar approach, Dinsmore and Klaenhammer[281] studied spontaneous mutants of the lactococcal phage ϕ31 (P335 species), which revealed insensitivity to the AbiA system. Rince et al.[267] have identified a 324 bp fragment containing the *cos* site from phage sk1 that reduced the effect of AbiF on phage 712.

More than one Abi system can be present in single lactococcal strains either on the same plasmid, such as Abi E and Abi F on pNP40 or on separate plasmids, such as AbiA and AbiC. Furthermore, Abi systems are frequently combined in a single lactococcal strain with other antiphage mechanisms. For example, five phage resistance mechanisms have been identified in *Lc. lactis* ME2 coded by the three plasmids pTR2030 (AbiA plus *Lla*I R/M system), pTN20 (AbiC plus another R/M system), and pME0030 (undefined phage adsorption inhibition mechanism).[230] The conjugative plasmid pNP40 encoding AbiE and AbiF also codes for a third mechanism inhibiting phage DNA injection.[228,277] The two genetic loci encoding AbiR mechanism flank the *Lla*KR2I R/M system on plasmid pKR223.[303] Abi systems are also supplemented by R/M systems in other lactococcal strains.[305,306] Thus, abortive infection only occurs in those cases in which a phage attack could not be blocked in previous steps of the lytic cycle of phage infection. Therefore, the incidence of cell death due to Abi systems is reduced to a minimum.

Response of phage resistance to temperature is of considerable practical importance, since elevated temperatures are routinely used during cooking in cheese manufacture. Thus, several Abi systems becomes significantly weaker at higher temperatures (40°C).[275,295,303] In contrast, phage-resistance mechanisms may become more active at higher temperatures in *S. thermophilus*.[307]

B. Artificial Phage-Resistance Mechanisms

Phage insensitivity of dairy starter strains can be conveniently improved by introducing naturally occurring phage-resistance determinants. However, it has been shown also that different elements from the phage itself can be exploited for phage control. Thus, phage genes have been cloned either with their own promoter or behind a new promoter, or genes have been cloned in antisense orientation behind new promoters. Also, noncoding phage sequences have been utilized for construction of novel antiphage mechanisms. Furthermore, a phage promoter has been combined with a lethal gene to construct a phage-triggered defense system.[308] Most of these systems are still not very efficient, and they only inhibit a small number of phages, most often only the phage from which the element has been cloned.[309]

1. Antisense RNA Strategies

One of these novel phage-resistance mechanisms is based on an antisense RNA strategy where a gene is cloned behind a promoter in its antisense orientation. It is anticipated that its antisense RNA transcript will bind to target sense mRNA, preventing translation either by destabilizing and making it more susceptible for degradation by RNases or by inhibiting loading of ribosomes.[310] In the first attempt, an undefined gene (*gp51c*) present

on a 1.6 kb fragment from lactococcal phage ϕ7-9 was cloned in the antisense orientation with respect to a strong lactococcal promoter.[311,312] This construct, when introduced into a lactococcal recipient, conferred significant phage insensitivity to the host cells (i.e., a 10^{-3} reduction in EOP accompanied by a reduction in plaque size). However, constructs based on truncated forms of the gene were less effective. Antisense constructs directed against other genes of ϕ7-9 (*gp18C*, *gp24C*) or the major capsid protein of another phage (ϕF4-1) were much less effective, implying that this technique requires care in selecting phage genes which have to be essential for phage proliferation and which should be transcribed at a low level.[313–315] Thus, two middle- and four late-expressed *orf*s from phage φ31 were cloned behind the strong *Lactobacillus* P6 promoter and the T7 terminator, but they had no effect on the EOP or plaque size of phage φ31. Only when the late-expressed gene was cloned on a vector containing the phage φ31 origin of replication some protection was obtained.[310] Successful exploitation of an antisense strategy was shown by McGrath et al.[128] They cloned eight different *orf*s from the phage Tuc2009 replication module as antisense constructs and found that *orf*17 and rep_{2009} efficiently inhibit proliferation of four P335 phages. Both rep_{2009} (the putative replisome organizer gene) and *orf*17 (the putative helicase loader gene) are involved in replication of phage Tuc2009.[128]

Many *S. thermophilus* phages possess a highly conserved DNA replication module, which was shown to be an effective target for an explosive antisense RNA strategy.[309] An antisense RNA cassette complementary to a helicase gene of a *cos*-site *S. thermophilus* phage was cloned into a phage-encoded resistance plasmid containing the origin of replication of the same phage. This combination resulted in a drastic reduction in efficiency of plaquing down to 10^{-7}–10^{-8}.

2. Utilization of Origin of Replication (PER)

Another approach is based on utilization of the phage origin of replication. The method was designated "Per" for **p**hage-**e**ncoded **r**esistance,[316] even though other kinds of phage elements may also be used for construction of antiphage mechanisms. When the cloned origin of replication (*ori*) of the lactococcal phage ϕ50[316] was introduced into *Lc. lactis* subsp. *lactis* NCK203, the host's insensitivity to ϕ50 infection was enhanced as indicated by a reduction in EOP and in plaque size. It was assumed that the cloned *per* locus competes in *trans* with normal phage replication. The Per system derived from phage ϕ50 was also effective against other phages (e.g., ϕ48) containing homologous phage origins of replication but failed to combat phages with different origins (e.g., ϕ31). Per systems have also been described for other lactococcal phages[62,317] and have been demonstrated to be effective in *S. thermophilus*[134,318] and *Lb. casei*.[135] Hence, as shown before for "artificial" mechanisms, this technique is also highly effective but specific for distinct types of phage.

3. Utilization of the Phage Repressor

Temperate phage have a gene encoding a repressor, CI, suppressing the expression of the lytic cycle. When *orf4* encoding the repressor from phage TP901-1 was cloned in pAK80, a middle copy number vector,[319] and transformed into the host *Lc. lactis* 3107, full protection against phage TP901-1 and an EOP less than 10^{-6} was observed.[173] When the homologous gene from the lytic P335 phage φ31 was cloned in pAK80 or pTRKH2 no inhibition was seen, but when a truncated version of the CI repressor was constitutively expressed on a high copy number vector, retardation

of several P335 phages was observed.[169] In *S. thermophilus* cloning of a repressor open reading frame from phage Sfi21 protected the strain against superinfection with homologous temperate phages, but not with virulent phages.[165] Integration of the *cI* gene of phage A2 into the *Lb. casei* host chromosome resulted in stable resistance against superinfection with the A2 phage. The engineered strain could be used for milk fermentation in the presence of viable A2 phage, which were eliminated from the milk through adsorption to the bacterial cells.[320]

4. Superinfection Exclusion

McGrath et al.[321] used a gene, designated sie_{2009} (superinfection exclusion), located between the genes encoding the repressor and the integrase on the temperate phage Tuc2009 to impede phage infections. Computer analysis predicted that the SIE_{2009} protein is associated with the cell membrane. They cloned the sie_{2009} gene downstream of a constitutive promoter on a high copy number plasmid, resulting in pNZ44 sie_{2009}. They found that *Lc. lactis* harboring the pNZ44 sie_{2009} did not protect the bacteria against phages belonging to the c2 or P335 species, whereas it confers a complete resistance phenotype against three 936 phages: sk1, jj50, and 712. It does not affect phage adsorption, transfection, and plasmid transformation, but interferes with plasmid transduction and phage replication, indicating that it blocks the injection of phage DNA like a superinfection exclusion mechanism. They screened DNA sequences of temperate phages for the presence of other phage-resistance mechanisms situated between the genes encoding the integrase and repressor, having a hydrophobic N-terminus and/or one possible membrane spanning domain. They found two such systems with similar phage-resistance phenotype to that conferred by sie_{2009}, but with no homology to sie_{2009}. They propose that superinfection exclusion mechanisms are widespread and that prophage sequences may contribute to the phage resistance of the bacteria.

S. thermophilus Sfi1 was transformed with the cloned superinfection exclusion gene of the temperate phage Sfi21 and showed resistance to superinfection by heterologous phage, but not to the homologous phage Sfi21.[322]

5. Phage-Triggered Defense Mechanism

This bacterial suicidal system employs a strictly phage-inducible promoter to activate a cassette of lethal genes.[308,323] A phage-inducible middle-expressed promoter from the lytic phage $\varphi 31$ was placed upstream of the lethal *Lla*IR restriction cassette consisting of three genes and cloned on a high copy number replicon. When phage $\varphi 31$ infected *Lc. lactis* with this construct, the EOP was lowered to 10^{-4} and the burst size was reduced fourfold.[308] It is very important that the phage promoter is tight and does not permit any transcription of the restriction endonuclease cassette in the absence of a phage infection. Since the system did not harbor the methylase gene, phages circumventing the mechanisms by methylation will not be developed. However, phages less sensitive to the mechanisms were developed after repeating exposure to it. These phages all had mutations in the gene encoding the activator of the phage promoter, thereby lowering the strength of activation. In general, this system requires a fine balance between the timing, the strength of the phage-inducible promoter, the tightness of its regulation, and the lethality of the selected suicide gene.[323]

IV. FROM CONVENTIONAL PHAGE CONTROL TO MODERN APPROACHES

Milk fermentations are not performed under aseptic conditions, since the substrate milk is usually treated by pasteurization and not by heat-sterilization prior to fermentation. However, phages are known to survive temperature conditions used for pasteurization,[192] and further, raw milk without any heat treatment is also quite commonly used for many traditional cheeses. Having once contaminated a dairy environment, phages are easily spread, in particular via the air and in whey residues of cheeses.[81,324,325] Thus, the steps used in handling whey are critical with respect to phage distribution (e.g., whey separators, whey drainage systems, filling lines). For economic reasons, milk fermentations have been continuously scaled up, and short-time fermentations with more than one filling of the vats per day are routine, enhancing the phages' opportunities to infect active starter bacteria continuously.

Lactococci harboring various plasmid-encoded phage-resistance systems have become a rich source of applications to improve the phage resistance of starter strains.[269,326–337] Many of these plasmids are conjugative, and hence conjugal strategies were developed avoiding the use of selecting transconjugants on the basis of antibiotic resistance markers in the recipient strains. Harrington and Hill[338] made use of a concomitant nisin resistance marker to transfer the phage insensitivity plasmid pNP40[339] by conjugation. Bacteriocin resistance suitable for selection is also a natural plasmid-encoded trait found to be linked with phage insensitivity determinants.[305,340–342] Similarly, lacticin 481[330] and lacticin 3147[343] have been used as selective markers to introduce phage resistance into starter cultures. Another method was the utility of cadmium resistance as a selective marker.[334] O'Sullivan et al.[336] have by sequential conjugal transfer using bacteriocin and cadmium resistance for selection constructed a strain harboring three different antiphage mechanisms: an adsorption inhibition mechanism, an R/M, and an Abi system.

Insensitivity plasmids have also been transferred by co-transformation, originally by protoplast transformation, but later most conveniently by electroporation.[231,292,306,344,345] Phage-resistance determinants were also routinely cloned into suitable vectors and subsequently used for transformation (e.g., Refs.[242,250,260,275,277,279,286,289,290,294,346–350]). As transformation of naturally occurring R/M-encoding plasmids into starter strains is not easily performed, it is advisable to evaluate compatibility between the incoming and resident plasmids and to determine the actual strength of phage protection against the disturbing phages of starter strains grown in milk before steps for improvement of starter strains are initiated. In order to facilitate the selection of transformed starter strains, a chloramphenicol resistance cassette was inserted into the native R/M-encoding plasmids. The potential of systems *Lla*AI and *Lla*BIII on plasmids carrying their original replicon to protect starter strains has been evaluated in this way.[331,332] Cloning of an Abi-type phage-resistance determinant in a food-grade vector using the natural nisin-resistance selection marker opens new perspectives for safe applications in dairy fermentations.[351,352] These various approaches also allowed the stacking of various phage insensitivity plasmids conferring complementary phage-resistance mechanisms to single strains in order to enhance the phage insensitivity to different phage types common in dairies.[353] Combining R/M systems with abortive defense mechanisms in lactococci also improves cell survival upon phage infection, since cell death due to phage-induced abortion will be minimized.[345,350]

It has also been demonstrated that cloned phage resistance determinants derived from the lactococcal plasmid pSRQ700, which expresses R/M activity (*Lla*DCHI), could also be expressed in the thermophilic species *S. thermophilus*.[347] The lactococcal abortive infection mechanisms AbiA and AbiG were also introduced into a *S. thermophilus* strain. AbiA proved effective at 30°C but not at 37 or 42°C.[354] AbiG did not affect any phages.[354] This success in crossing the species barrier will undoubtedly open new perspectives in enhancing phage resistance of nonlactococcal starter strains.

V. PHAGE COUNTERDEFENSE STRATEGIES

Phages that are continuously exposed to bacterial phage defense mechanisms will develop counterresistance mechanisms. In general, phages can avoid restriction by decreasing the number of restriction sites in their genome.[12,355,356] Furthermore, phages may acquire a functional part of the structural gene encoding a methylase.[203] As mentioned above, lactococcal phages able to prevail over different Abi systems have been isolated. In general, phages overcome Abi systems either by introduction of point mutations or by recombination with other phages or prophages in the genome of the host.[267,281,293]

VI. PHAGES AS GENETIC TOOLS

A. Transduction

Phages may contribute efficiently to vertical gene fluxes among their bacterial hosts by transduction, and both virulent and temperate phages are known to mediate the transfer of either chromosomal or plasmid DNA among strains of lactococci,[357–365] lactobacilli,[358,366] and *S. thermophilus*.[367] Transduction was also confirmed in the milk matrix.[368] Transduction transfer efficiencies of vector constructs increased significantly upon insertion of phage-specific genome sequences as shown for the *cos*-site regions of the temperate lactococcal phage ϕLC3 and of the virulent phage c2 cloned into the shuttle vector pSA3.[139,369] Recently it was shown that transduction of plasmids containing the *cos* region of the lactococcal phage sk1 occurred by packaging of trimeric concatemers terminating in the *cos* ends.[370] Enhanced plasmid transduction of the lactococcal plasmid vector pGK12 by the temperate *Lb. gasseri* phage ϕadh was also achieved by inserting randomly cloned ϕadh DNA into pGK12. The increase in transduction frequency of the recombinant plasmids correlated with the extent of DNA-DNA homology between these constructs and phage ϕadh. This strategy also allowed a transductional transfer into *Lb. gasseri* strains which did not support lytic growth of phage ϕadh.[371] Transduction has been successfully applied for detailed host range determinations of temperate lactococcal phages and provided a good methodology for rapidly testing strains for both the ability to adsorb phages and permit DNA injection.[321,369,372]

B. Construction of Site-Specific Integration Systems

During recent years, research has focused on the temperate phage of lactic acid bacteria in order to exploit their capacity to promote site-specific integration into bacterial. For a first-generation type of integration vector, undefined lactococcal prophage DNA was cloned into a suitable vector (pE194) unable to replicate in lactococci.[373] Later, site-specific phage integration cassettes derived either from the temperate lactococcal phages ϕLC3,[374] TP901-1,[178,184] Tuc2009,[375] TPW22,[182] from temperate *Lactobacillus*

phages ϕadh,[376,377] mv4,[177] A2,[186] ϕFSW,[16] from temperate *S. thermophilus* phage Sfi21,[183] and from temperate *Oenococcus* phage ϕ10MC[185] were used to construct integration vectors. The phage-derived cassettes include the corresponding phage attachment sites (*attP*) and the adjacent phage integrase genes, respectively, and were cloned into suitable nonreplicative vectors. Integration occurred by a site-specific recombination process between the *attP* of the phage genome and the homologous *attB* site on the prophage-cured host chromosome following the Campbell model.[378] The integration vector pBC170 based on the integration system of the lactococcal phageTP901-1[178] integrated into the lactococcal chromosome very efficiently (8×10^5 transformants per μg DNA). When a DNA cassette containing the integrase gene from the lactococcal phage ϕLC3 and its *attP* site were inserted into a replication-thermosensitive plasmid, essentially all transformants obtained from *Lc. lactis* subsp. *cremoris* LM0230 were shown to be integrants. Due to the high efficiency of transformation plus integration events, DNA constructs directly obtained from ligase reaction mixtures could be used for site-specific integration.[374]

Since *Lb. delbrueckii* subsp. *bulgaricus* is recalcitrant to transformation techniques, insertion of an integration vector based on the *Lb. lactis* subsp. *bulgaricus* phage mv4 was demonstrated in the heterologous host *Lb. plantarum*, since a homologous *attB* site required for integration was also present in the heterologous host.[177] The site-specific integration of the phage mv4–derived integration vector pMC1 into the chromosomes of various other bacteria was shown later (i.e., *Lb. casei, Lc. lactis* subsp. *cremoris*, *Enterococcus faecalis*, *S. pneumoniae*), illustrating the wide host range of this phage-derived integration system.[379] This flexibility of phage integrases was also noted for *Lb. casei* phage A2[186] and for lactococcal phage TP901-1,[179] and recombination could also take place in *E. coli.* The TP901-1 integration system is even functional in mammalian cells.[380] Integrases of phage mv4 and of phage TP901-1 did not require additional bacterial host factors for recombination.[179,381] The TP901-1 integration system was used to construct chromosomal single-copy transcriptional fusions on basis of promoter-reporter vectors.[382]

A unique site-specific integration and delivery system based on the integrase gene and the *attP* site of the *Lb. casei* phage A2 has been combined with a heterologous β-recombinase–derived clearing system. This two-component system allowed the safe deletion of non–food-grade genes required for selection during the integration process.[383]

C. Inducible Gene Expression Systems

Based on the genetic switch elements of the temperate *Lc. lactis* phage r1t, a food-grade inducible gene expression system has been constructed for lactococci. The system included the two divergently orientated genes *rro* (putative phage repressor: repressor of r-one) and *tec* (topological equivalent of the phage λ *cro* gene), respectively, their preceding promoters, and three 21 bp operator sites, two of which partially overlap the promoter sequences.[167] The intact regulatory region was placed in front of a *lacZ* (*E. coli*) translational fusion constructed with an ORF following *tec.* Hence, the activity of the translational fusion construct became controllable by addition of mitomycin C. Since this agent promotes the switch of the temperate phage from the lysogenic to the lytic cycle, expression of the *lacZ* fusion could be induced 70-fold. Later, a temperature-sensitive derivative of the phage r1t repressor was designed for the development of a more efficient temperature-inducible expression system.[384] The *Lb. casei* phage ϕFSW was used as

source for isolation of the thermoinducible promoter-repressor cassette. In the lysogenic *Lb. casei* host, a *gusA* reporter gene under the control of the isolated ϕFSW promoter was repressed at 28°C and expressed at 42°C.[385]

D. Exploitation of Phage-Encoded Lysins

It has been shown for *Lc. lactis* and *S. thermophilus* starter cultures that their autolytic behavior correlates well with the presence of prophage DNA in their chromosomes.[386–388] These strains contribute to an accelerated cheese ripening and debittering process due to the release of peptidolytic enzymes.

Phage-encoded determinants for cell-wall–degrading enzymes (lysins) were among the first genes to be cloned from phages of lactococci[389] and lactobacilli.[150] Expression of the cloned lysin gene of the lactococcal phage ϕvML3 in lactococci resulted in cell lysis in the stationary phase.[390]

The lysis cassettes of the lactococcal phages r1t[391] and ϕUS3[392] were used in food-grade chloride or nisin-inducible gene expression systems for controlled accelerated cheese ripening. Expression of the transcriptional activator (Tac31A) of the lactococcal phage ϕ31 in *trans* in lysogenic strains harboring a phage ϕ31-homologous promoter resulted in a leaky behavior of these cultures due to the activation of the holin-lysin cassettes on the resident prophages. Integration of the phage r1t lysis cassette under the control of the phage ϕ31 late promoter into a lactococcal host chromosome resulted in a similar leaky phenotype.[393] Growth of the leaky cells, which could externalize enzymes and antigens without the use of export signals into the growth medium, was not severely affected.

Vasala et al.[394] have shown that the lysin of the *Lb. delbrueckii* subsp. *lactis* phage LL-H also hydrolyzed cell walls of other lactic acid bacteria (*Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, *Lb. helveticus*, *Pediococcus damnosus*), which may enhance its possible role as a cheese-ripening additive. The LL-H lysin cloned can easily be purified from *E. coli* cells by thymol treatment and expanded-bed adsorption chromatography.[395,396] A purified phage-encoded lysin has been exploited to generate protoplasts suitable for transfection studies of *Lb. casei* ATCC 27092.[397,398]

E. Phage-Induced Plasmid Amplification and Gene Expression

O'Sullivan et al.[317] have shown that the presence of the origin of replication from phage ϕ31 (Per31), when cloned in *trans* on a plasmid, can promote a significant amplification of the plasmid construct upon superinfection of the lactococcal host with the homologous phage ϕ31. Based on this observation, a promoter-screening strategy was used to fuse ϕ31-specific expression signals (i.e., middle inducible phage promoters) with a promoterless *lacZ* gene from *S. thermophilus.* This ϕ31 promoter *lacZ* cassette was subsequently cloned in a low copy vector containing the *Per31* locus.[399] Infection of *Lc. lactis* NCK203 harboring this construct resulted in an explosive induction of β-galactosidase activity due to the synergistic effect of phage-induced gene expression and phage-induced plasmid amplification.

VII. CONCLUSIONS

During food fermentations, the handling of starter cultures composed of lactic acid bacteria cannot be performed under strictly aseptic conditions (as practicable in other

areas, e.g., the medical field). Hence, phage populations have ample opportunity to infect these cultures. Traditional and new strategies to keep the incidences of phage infections low rely on a deep knowledge of the dynamic relationships among the cultures and their viral enemies. Research on the phage has also opened a variety of different perspectives with respect to the genetic engineering of lactic acid bacteria.

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9

Mathematical Modeling of Intestinal Bacteria–Host Interactions

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I. INTRODUCTION

The beneficial effects of probiotics arise from the interactions between the probiotic bacteria, the host tissue, and the pathogens. Competitive exclusion of pathogens and immuno-modulation may involve interaction between the adhesins on the bacterial surface and the adhesion sites (in hydrophobic interaction) or specific receptors (in ligand-receptor interaction) on the host cells.[1] This interaction is a dynamic process, and appropriate interpretation of the kinetics would yield useful insight into the adhesion mechanisms for the development of strategies to maximize probiotic effects for specific applications.

II. MATHEMATICAL MODELING

A. Adhesion Kinetics

The displacement and exclusion of pathogens from the intestinal tract in human and animal models[2] suggest that the adhesion of bacterial cells on intestinal mucosal surface is a reversible process. In vitro studies by incubating bacterial cells with cell lines, mucin glycoprotein, and tissues have demonstrated that adhesion of bacterial cells on the intestinal surface at equilibrium is bacterial strain and cell density dependent.[3–5] Fig. 1 shows a hyperbolic relationship between the concentration of adhered bacterial cells and the time of incubation with the bacteria suspension. The bound bacterial concentration at equilibrium was a function of the bacterial concentration in the suspension, clearly demonstrating that the adhesion of bacteria on the intestinal surface is a reversible and dynamic

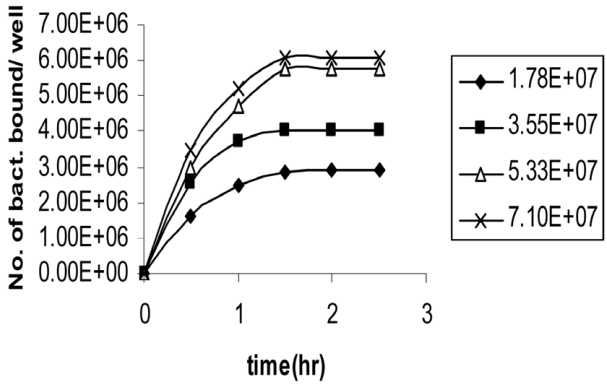

Figure 1 Plots of the concentrations of bound *Lactobacillus rhamnosus* on enterocyte-like Caco-2 cells measured at various time of incubation. The data show that the equilibrium concentrations of bound bacteria determined at 1.5 h incubation was a function of the bacterial concentration in suspension (between 1.78 and 7.10×10^7 cells/mL).

process. In an irreversible adhesion kinetic, all receptors on the intestinal surface will eventually be bound and the equilibrium adhesion concentration is independent of the bacterial density in the suspension (Fig. 2). In such a case, the turnover of bacterial cells on the intestinal surface depends on the turnover of the mucosal layer and recolonization of newly exposed surface.

The hyperbolic relationship shown in Fig. 1 suggests that the adhesion process of bacterial cells on intestinal surface receptors is a simple dissociation kinetic. The Langmuir adsorption isotherm,[6,7] Scatchard,[8,9] and Michaelis-Menten–type dissociation kinetic models[4] have been used to describe adhesion kinetics:

1. Langmuir equation (Fig. 3):

$$X/e_x = 1/(K \cdot e_m) + X/e_m$$

or

$$1/e_x = 1/(K \cdot X \cdot e_m) + 1/e_m$$

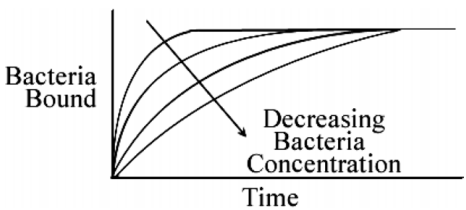

Figure 2 In an irreversible adhesion kinetic, all receptors on a surface will eventually be bound and the equilibrium adhesion concentration of bacteria is independent of the bacterial concentration in the suspension.

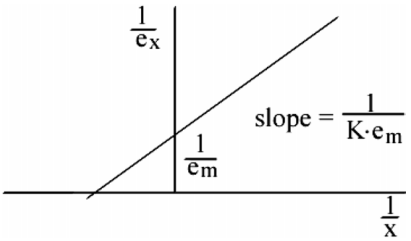

Figure 3 Plot of the Langmuir equation: x = added cell concentration; e_x = bound cell concentration; e_m = maximum bound cell concentration (numerically equal to the concentration of receptors); K = kinetic constant.

where

X = added cell concentration
e_x = bound cell concentration
e_m = maximum bound cell concentration (numerically equal to the concentration of receptors)
K = kinetic constant

2. Scatchard equation (Fig. 4):

$$e_x/X = K \cdot e_m - K \cdot e_x$$

3. Dissociation kinetic model (Fig. 5)

$$1/e_x = 1/e_m + K/(e_m \cdot X)$$

These physiological models allow the incorporation of physiological parameters in the kinetic models. The working principles and derivations of the last dissociation kinetic model will be discussed in detail in this chapter.

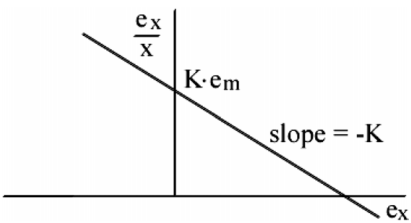

Figure 4 Plot of the Scatchard equation: x = added cell concentration; e_x = bound cell concentration; e_m = maximum bound cell concentration (numerically equal to the concentration of receptors); K = kinetic constant.

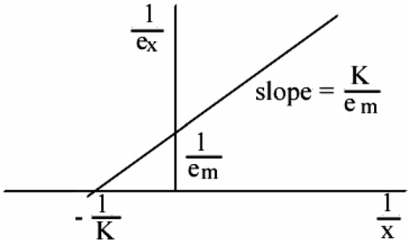

Figure 5 Plot of simple dissociation kinetic: x = added cell concentration; e_x = bound cell concentration; e_m = maximum bound cell concentration (numerically equal to the concentration of receptors); K = kinetic constant.

In a process of simple dissociation involving bacteria and the intestinal surface (which includes the mucus layer and the epithelial cells):

$$\text{Bacterial cell} + \text{Intestinal cell} \underset{K-1}{\overset{K+1}{\rightleftarrows}} \text{Bacterium–Intestinal cell complex}$$

where K + 1 and K − 1 represent the association and dissociation constants of the reaction, respectively. The process is similar to the interaction between a substrate and the receptor on an enzyme that forms a substrate-enzyme complex, but without the subsequent formation of products.

There are two assumptions in the relationship:

1. It is assumed that the interaction between the bacterial cells and the intestinal cells or mucus remains in equilibrium. This condition should be achieved if the bacterial cells do not penetrate the intestinal cells.
2. It is also assumed that the concentration of the bacterial culture remained essentially unchanged throughout the study, so that the concentration of the bacterial culture can be considered equal to the initial bacterial concentration. This condition could be achieved when the total number of bacterial cells is much greater than the number of bacterial cells adhering to the intestinal cells. This is usually the case in most of the adhesion studies, where the concentration of the bacterial cells added is in the range of 10^5–10^8 per mL, whereas that of the intestinal cell culture (e.g., Caco-2 cells) is about 10^2 per mL, and the number of bacterial cells adhering to the Caco-2 cells fewer than 10 per cell. In the intestinal tract, the initial concentration of the bacterial cells (probiotics) after their intake is maintained for a period of time. After that, the luminal bacterial cells are washed out by food and drink. The local concentration of a bacterium around a receptor site is determined by the rate of cell division of those adhered onto the mucosal surface.

In the simple dissociation equation described above, if X is the concentration of the bacterial culture added, e is the initial epithelial cell or mucus concentration, and e_x is the concentration of the bacterium-intestinal cell-mucus complex, then the concentration of free epithelial cells or mucus will be $(e - e_x)$.

Because the process is in equilibrium, the dissociation constant for the process (K_x) can be defined as:

$$K_x = (K - 1)/(K + 1) = (e - e_x) \cdot X/e_x$$

This equation can be rearranged to give an expression for the concentration of the bacterium–intestinal cell–mucus complex:

$$e_x = e \cdot X/(K_x + X)$$

When X is very much larger than K_x, the intestinal cells or mucus is saturated with bacteria (i.e., e_x approaches e) and the maximum value of e_x, e_m is obtained. As it is technically easier to estimate the maximum concentration of adhered bacterial cells (e_m) than the epithelial cell/mucus concentration (e), the equation could thus be re-written as:

$$e_x = e_m \cdot X/(K_x + X) \quad (1)$$

The equation could be further rearranged to give a linear relationship:

$$1/e_x = 1/e_m + K_x/(e_m \cdot X) \quad (2)$$

Hence, plots of $1/e_x$ against $1/X$ give straight lines (Fig. 6), in which the intercepts on the ordinate give the values of $1/e_m$ and those on the abscissa give the values of $-1/K_x$ (Table 1). The values of e_x and K_x are independent of each other, i.e., a bacterium that

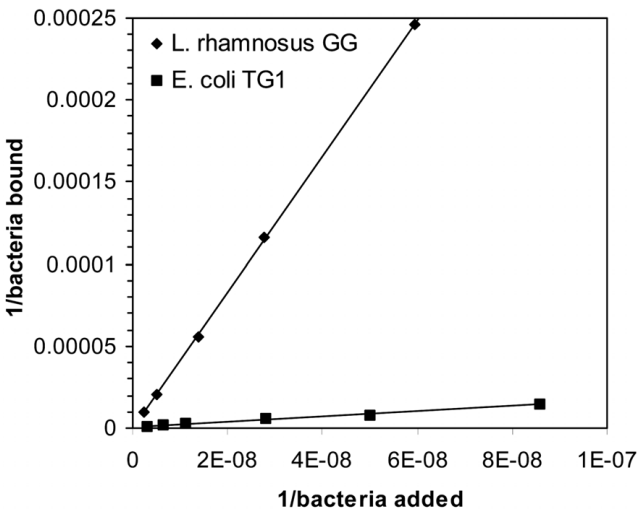

Figure 6 Double reciprocal representation of the adhesion of *Lactobacillus rhamnosus* GG and *Escherichia coli* TG1 to immobilized human intestinal mucus. The lines indicate the linear fit according to the least-squares method.

Table 1 Maximum Concentration of Bound Bacterial Cells on Enterocyte-Like Caco-2 Cells (e_m) and the Kinetic Constant of the Adhesion Process (k_x).

Strain	e_m (cells/100 Caco-2 cells)	k_x (cells/mL)
L. rhamnosus GG	1613	2.08×10^8
E. coli TG1	500	4.76×10^8

Values were calculated from Eq. (2) based on data presented in Fig. 6.

adheres on the intestinal surface in large numbers could have a low affinity for the intestinal surface receptors and vice versa. This has two practical implications:

1. The readout from a single point (using only one bacterial cell concentration) evaluation of the adhesiveness of a bacterium on the intestinal surface is determined by the bacterial cell concentration tested. In the case demonstrated in Fig. 7, probiotic strain A has lower K_x value but higher e_m value than probiotic strain B. At bacterial cell concentration 1 (as shown on the x-axis), strain B showed higher adhesiveness than stain A, but at concentration 2 the reverse was observed. In the intestinal environment when strain A or B was first given to a human subject, larger numbers of strain A would be found on the surface of the intestinal tract than of strain B, because the saturating bacterial cell concentration was near point 2 in Fig. 7. As time went on, the luminal bacterial concentration was diluted by food and water consumed. In this case, strain A was washed out from the intestinal surface at a faster rate than strain B, because strain B had a higher affinity for the intestinal surface receptors and did not detach as readily as strain A. The selection of the appropriate probiotic strains would depend on the intended probiotic effects. For example, for short-term flooding of the intestinal surface with a high concentration of probiotic bacterial cells to exclude or displace pathogens, A-type probiotic strains are desirable. If the desired effect is longer adhesion of the probiotic bacterial cells on the intestinal surface to induce immunological responses, B-type probiotic strains would be the better choice.
2. A question often touched on in the study of adhesion of bacteria to the intestinal surface is whether a nonsaturating or saturating concentration of the bacteria should

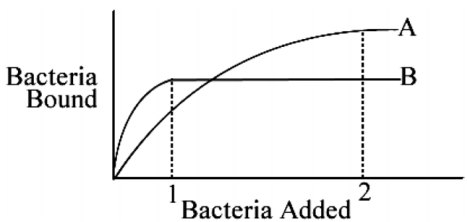

Figure 7 Correlation between the concentration of bound bacterium A and B and the concentration of the respective bacterium in suspension. Bacterium A shows higher adhesion affinity for the surface but lower maximum adhesion concentration than bacterium B.

be used. The two approaches in fact yield different information. Nonsaturating bacterial cell concentrations largely reflect the affinity for the receptors, whereas saturating bacterial concentrations reflect the concentration of the receptors on the intestinal surface.

B. Competition for Adhesion

When two types of bacteria are present and compete for the same receptors or adhesion sites (through steric hindrance of cells in close vicinity) on the intestinal surface, the competition for adhesion of each bacterial type is determined by the affinity of the bacteria to the intestinal surface (K_x) and the concentration of the bacterial cells (X). Thus, the ratio of e_x for bacterium 1 and bacterium 2 in the mixed bacterial system can be described as:

$$e_{x1}/e_{x2} = (e_{m1}/e_{m2}) \cdot (X_1/X_2) \cdot [(K_{x2} + X_2)/(K_{x1} + X_1)] \quad (3)$$

The relationship was verified in a study on the competition between *Lactobacillus rhamnosus* GG and *Escherichia coli* TG1 for adhesion on enterocyte-like Caco-2 cells (Table 2).[4]

The relationship in Eq. (3) suggests that the outcome of competition between two bacteria for adhesion to the same receptors on the mucosal surface is determined by the ratio of the respective bacterial concentration around the receptors and the affinity of the respective bacterium for the receptors. High bacterial cell concentration would ensure frequent encounter between the bacterial cells and the receptors, thus a high chance for the bacterium to adhere onto the receptor sites. Such a bacterium would need to grow and divide rapidly in the intestinal environment in order to maintain a sufficiently high local cell concentration after the external (oral) supply has stopped. A bacterial cell that has a high affinity for the receptor will not dissociate readily enough to be replaced by another bacterial cell. Thus, one may select for a probiotic bacterium with very high affinity for the intestinal receptors to prevent from being outcompeted by other bacteria and washed out from the intestine. However, such as bacterium would not be released readily from the dislodged mucosal layer and would be discharged as fecal material.

Table 2 Competition for Adhesion of *L. rhamnosus* GG and *E. coli* TG1 on Caco-2 Cells in a Mixed Suspension of the Two Bacteria

	L. rhamnosus added (cells/mL)		
	1.0×10^8	2.0×10^8	3.0×10^8
Observed e_E/e_L	0.128	0.074	0.038
Predicted e_E/e_L	0.166	0.071	0.041

e_E = Concentration of adhered *E. coli* TG1 on 100 Caco-2 cells; e_L = Concentration of adhered *L. rhamnosus* GG on 100 Caco-2 cells. The predicted values of e_E/e_L were calculated from Eq. (3).

C. Heterogeneity of Receptors and Adhesins

Heterogeneity in the adhesin-receptor interaction is expected due to the minor differences among the many receptors on intestinal surface and adhesins on a bacterial cell. The adhesion kinetics could still be described by Eq. (2), with the ratio of the different adhesin-receptor interactions determined by Eq. (3). In this case, the overall affinity of the interactions is the weighted average, as follow:

$$K_x = (e_{x1}/e_x) \cdot k_{x1} + (e_{x2}/e_x) \cdot k_{x2}$$

where:

$$\begin{aligned} e_x &= e_{x1} + e_{x2} \\ e_{x1}/e_{x2} &= (X_1/X_2) \cdot [(k_{x2} + X_2)/(k_{x1} + X_1)] \\ e_{m1} &= e_{m2} \end{aligned}$$

However, bacterial cells with lower affinity for the receptors will be washed out (detached) faster than those that have higher affinities. In a study where adhered *E. coli* cells were subjected to a continuous flow of nutrient, a precipitous drop in the number of adhering bacteria was first observed, followed by a more gentle decrease in the number of surface-adhering bacteria.[10] This was attributed to two types of adhesion sites for the *E. coli* mediating weak or strong adhesion, respectively.

D. Deviations from the Simple Dissociation Kinetic

1. Cooperative Effect

If binding of a bacterium on the mucosal surface changes the affinity for the subsequent bacteria binding (either on the same or adjacent intestinal cells or mucus surface), a cooperative effect (negative cooperative if the binding affinity decreases and positive if the affinity increases) is observed. This could be due to the modification of the physical configuration of the neighboring receptor after a bacterium is bound onto the first receptor site; it could also be due to auto-adhesion of adjacent bound bacteria. The plots of $1/e_x$ vs. $1/X$ would appear as curves (Fig. 8). The cooperative effect could be modeled by means

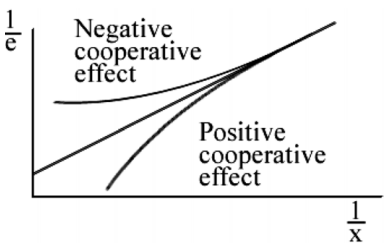

Figure 8 Kinetics of cooperative effects on the adhesion of bacteria on a surface: x = added cell concentration; e_x = bound cell concentration.

of the Hill equation.

$$\underset{n \cdot X}{n \text{ number of bacteria}} + \underset{(e - eX_n)}{\text{Intestinal cell}} \overset{k_h}{\rightleftharpoons} \underset{eX_n}{\text{Bacteria–Intestinal cell complex}}$$

The Hill constant, $k_h = (e - eX_n) \cdot (x^n)/(eX_n)$, where $e = (k_h \cdot eX_n/X^n) + (eX_n)$. At equilibrium, $eX_n/eX_m = eX_n/e$, where eX_m is the maximum concentration of bacteria–intestinal cell complex:

$$\begin{aligned}
eX_n &= eX_m \cdot eX_n/e \\
&= eX_m \cdot eX_n \cdot [X^n/(k_h \cdot eX_n + X^n \cdot eX_n)] \\
&= eX_m \cdot [X^n/(k_h + X^n)] \\
eX_n \cdot k_h + eX_n \cdot X^n &= eX_m \cdot X^n \\
eX_n \cdot k_h &= eX_m - eX_n \cdot X^n \\
&= X^n \cdot (eX_m - eX_n) \\
[eX_n/(eX_m - eX_n)] &= X^n/k_h
\end{aligned}$$

Thus,

$$\log[eX_n/(eX_m - eX_n)] = n \cdot \log X - \log k_h$$

The values of eX_n and eX_m could be determined experimentally by varying the concentration of X. The plot of $\log[eX_n/(eX_m - eX_n)]$ vs. $\log X$ would yield a linear relation with the slope numerically equal to n, and the intercept on the x-axis $\log k_h$ (Fig. 9).

Note that autoadhesion of probiotics may show a pseudo-positive cooperative effect. There are reports that some probiotic bacterial cells could adhere to each other and form aggregates.[11,12] Assuming that a bacterial cell could bind to two other bacterial cells, thus binding a bacterial cell (either to the mucosal receptor or to the bound bacterial cell), would lead to binding of two more bacterial cells, giving the impression that binding of a bacterial cell results in the exponentially increased number of bacterial cells that could bind onto the mucosal surface. In such a case, an infinite number of bacterial

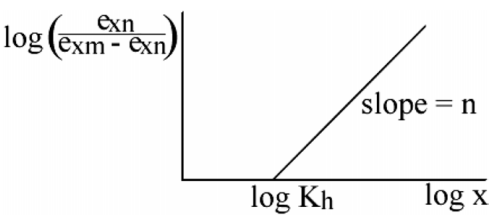

Figure 9 Plot of Hill equation for cooperative effect in the adhesion of bacteria on a surface: x = added cell concentration; e_{Xn} = bound cell concentration; e_{Xm} = maximum bound cell concentration (numerically equal the concentration of receptors); K_h = Hill constant; n = number of receptors on the surface.

cells could bind onto the mucosal receptors, and the plot of $\log[eX_n/(eX_m - eX_n)]$ vs. log X approaches a near-vertical slope.

2. Alternative Adhesion Interactions

Unrelated adhesins and hydrophobic surfaces can be found on the same bacterial cell. For example, both mannose-sensitive adhesin and hydrophobic adhesive surface are present on the surface of lactobacilli.[13] The two independent sets of adhesion interactions could be described as:

$$e_x = e_{m1} \cdot [X/(k_{x1} + X)] + e_{m2} \cdot [X/(k_{x2} + X)]$$
$$e_m = e_{m1} + e_{m2}$$

The mannose-sensitive adhesins could be masked by the inclusion of sugars. In a study on *Lactobacillus casei* Shirota, the affinity of the adhesins for mannose-mediated receptors on enterocyte-like Caco-2 cells was found to be 2.6 times that of the hydrophobic surface interaction between the bacterium and Caco-2 cells.[13] It was found that $e_{m1} = e_x = e_{m2}$, suggesting that the adhesins and the hydrophobic surfaces on *L. casei* Shirita are in close proximity and have the same number of adhesion sites. For example, the adhesin is located at the tip of a fimbria, whereas the stalk of the fimbria is hydrophobic. In this case, $e_x = e_m \cdot [X/(k_{x1} + X)]$ or $e_m \cdot [X/(k_{x2} + X)]$, whichever is higher: $e_{m1} = e_x = e_{m2}$.

3. Binding of a Bacterial Cell to Multiple Receptors

More than one adhesin or adhesive surface on a bacterial cell may bind onto the adjacent receptor sites at the same time. The size (surface area) of bacterial cells has been shown to correlate to their adhesion property.[14] When adhesion of a bacterial cell on the intestinal mucosal surface involves more than one adhesion receptor, it is envisaged that the kinetic constants determined at high and low cell concentrations would be different. At low bacterial cell concentration, the adhesion of a bacterial cell on the intestinal surface involves the maximum number of adhesion receptors (Fig. 10). At high bacterial cell concentration, there is self-competition for adhesion and the minimum number of receptors is involved. Thus, in the plot of $1/e_x$ vs. $1/X$, two linear plots are expected at high and low bacterial concentrations, with a curve between, representing the involvement of various numbers of receptors. In a study on the adhesion of *L. casei* Shirot on human intestinal mucus layer, two straight lines were observed in the plot of $1/e_x$ vs. $1/X$ (Fig. 11).[4] The higher dissociation constant ($k_x = 9.47 \times 10^7$ cells/mL) measured at high *Lactobacillus* concen-

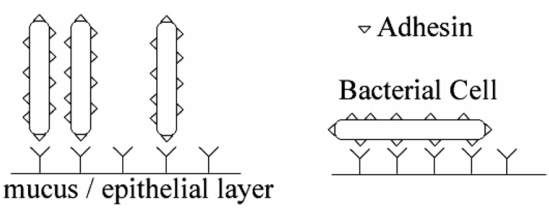

Figure 10 Schematic representation of adhesion of bacterial cells at high and low concentrations on an intestinal surface.

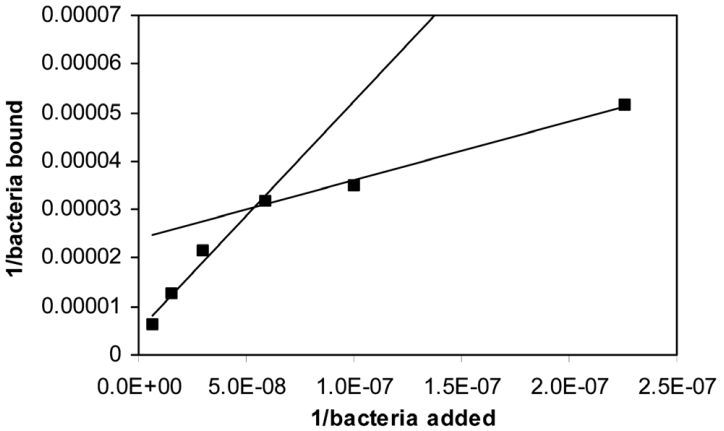

Figure 11 Double reciprocal representation of the adhesion of *Lactobacillus casei* strain Shirota to immobilized human intestinal mucus. The lines indicate the linear fit according to the least-squares method.

trations compared to that observed at low cell cencentrations ($k_x = 6.10 \times 10^6$ cells/mL) is likely to be due to the fourfold lower number of receptors ($5 \times 10^4/20 \times 10^4$ cells/well) involved. This suggests that the optimal concentration of *L. caeei* Shirota for competitive exclusion of a pathogen competing for the same receptors would be the concentration that allows the binding of the maximum number of adhesins on a bacterial cell. A relatively low concentration of the probiotic lactobacilli could occupy all the receptor sites on the intestinal mucosal surface, and the affinity for the receptors is the highest.

E. Colonization on the Intestinal Mucosal Surface

No probiotics used in clinical trials and commercial production have been shown to persist in fecal samples for more than a few weeks after their administration ceased.[15–18] This effect is termed colonization resistance. The ability of exogenously administered probiotics to adhere to the intestinal surface and multiply in the intestinal tract has been questioned.[19] Other reports on the recovery of lactobacilli from human colonic biopsies after discontinuation of probiotic administration[20–22] provide direct evidence that probiotic lactobacilli are able to temporary colonize colonic mucosae. These probiotic lactobacilli were selected for their high adhesiveness to the mucosal surface, fast growth rate, and acid and bile tolerance. Despite their demonstrated ability to displace indigenous bacteria and colonize the intestinal surface temporarily when first administered,[2] they are not able to colonize the intestinal surface permanently. Prolonged adhesion and colonization of probiotic bacteria on the intestinal mucosal surface could favour probiotic effects.

The process of washing out of an adhered bacterium from the intestinal mucosal surface can be described by the following relationship:

$$\text{Washout rate} = \text{Specific growth rate} - \text{Dilution rate}$$

The effective dilution rate is the balance between the rate of dilution of intestinal water overlying the mucosal surface by water and food consumed and the ability of the bacterium to withstand its removal from the mucosal surface. In order to persist on the mucosal surface (i.e., washout rate = 0), the bacterium would need to grow and divide at an average specific growth rate that equals the effective dilution rate. In a mouse model it was estimated that in order to permanently colonize the duodenum, jejunum, ileum, and colon, *L. casei* Shirota would need to attain a doubling time of 2.04, 1.17, 1.03 and 1.72 days, respectively, in the various sections of the intestine.[24] The doubling time of *L. casaei* Shirota in mouse duodenum, jejunum, ileum, and colon was 4.10, 4.78, 4.56, and 5.59 days, respectively. This study suggests that besides direct competition between intestinal bacteria for adhesion, the rates of growth and division determine the ability of probiotics to colonize and persist on the intestinal surface. The high concentration of probiotic bacteria at the time of consumption increases their chance of gaining a foothold on the intestinal surface. The rate of cell division determines the local bacterial cell concentration that allows the bacteria to persist on the intestinal surface. An adhered bacterium that is unable to divide under the intestinal conditions would be displaced by neighboring dividing indigenous bacteria, which are present in large concentrations around the receptor, according to Eq. (3).

III. CONCLUSION

Appropriate construction of mathematical models for microbe-host interactions demands identification of the key parameters involved. This helps in the design of experimental studies to verify their involvement and in the quantitative evaluation of their impact. Verification of mathematical models has yielded valuable insights into adhesion mechanisms.

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10

Methods of Analyzing Gut Microbiota

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I. INTRODUCTION

The human gastrointestinal tract harbors a complex collection of microorganisms, which form a specific individual microbiota for each person. This specific microbiota is dependent on the environment and genetic factors. The total number of microbes in the intestinal tract can be estimated at 10^{12} bacteria/g of intestinal contents. Several hundred bacterial species can be identified using traditional culture methods. The use of molecular methods has increased the number of bacteria in the intestinal mucosa and intestinal contents and also changed our understanding of the composition of the intestinal microbiota. The microbiota is metabolically active, and its composition is related to many disease states within and outside the intestine.

The intestinal microbiota provides the most important contact with the environment for the host and a barrier against harmful food components and pathogenic bacteria.[1–3] The intestinal microbiota has also been shown to have a direct impact on the morphology of the gut.[4] Therefore, the intestinal microbiota is an important factor for the health and well-being of the human host. Many diseases and their prevention can be linked to intestinal microbiota. The healthy microbiota may also be considered a good source of future probiotics.

II. DEVELOPMENT OF INTESTINAL MICROBIOTA

The basis of healthy gut microbiota lies in early infancy and initial development of intestinal colonization. The generation of immunophysiological regulation in the gut also depends on the establishment of indigenous microbiota. The microbiota of a newborn develops rapidly after birth and is initially strongly dependent on the mother's microbiota,

mode of delivery, and birth environment and is subsequently influenced by infant feeding practices and the environment of the child. Most microbiota succession studies have been based on culture method studies. Recent molecular studies have indicated that the microbiota in infants develops rapidly during the first week and remains relatively unstable for the first year of life, with changes occurring during breast feeding, weaning, and introduction of solid foods. Thus, healthy microbiota can be defined as the normal individual microbiota of a child that both preserves and promotes well-being and absence of disease, especially in the gastrointestinal tract. Healthy microbiota also provides the first step in the long-term well-being later in life, and the basis for this development lies in early infancy. Later in life the microbiota forms a barrier between the human host and the environment, providing the host resistance towards pathogenic bacteria and other harmful components in the environment. In adults the microbiota appears to be relatively stable, but among the elderly the microbiota approaches a state observed in infants and variations may be linked to ageing and specific diseases.

III. IMPORTANCE OF UNDERSTANDING INTESTINAL MICROBIOTA

It is obvious that understanding the cross talk that occurs between intestinal microbiota and its host promises to expand our views about the relationship between intestinal microbiota and health. There is also an increasing amount of information indicating that specific aberrations in intestinal microbiota may make us more vulnerable to intestinal inflammatory diseases and other diseases beyond the intestinal environment. It is likely that some aberrations even predispose us to specific diseases. Unfortunately, we are still far from knowing the qualitative and quantitative composition of the intestinal microbiota and which factors govern its composition in an individual. Several different methodologies could be used and further developed for intestinal microbiota assessment, categorized as either culture-dependent or culture-independent procedures.

IV. CURRENT METHODOLOGIES FOR MICROBIOTA ASSESSMENT

A. Culture-Dependent Techniques

1. Culture in Selective-Differential Media

The study of intestinal microbiota, both qualitative and quantitative, has been carried out mainly by means of culture in different selective and differential media of fecal microbiota as a measure of the bacterial contents in the colon. The classical method involves culturing fecal samples on suitable growth media. The sample is diluted, plated on a specific media, and the bacterial counts determined after incubation in the appropriate conditions. Bacterial strains can be isolated from these plates for further study or characterization. Usually isolated strains were identified by means of their phenotypic characteristics, mainly carbohydrate fermentation profiles, leading to numerous misidentifications. The development of genetic methods such as ribotyping, pulsed-field gel electrophoresis (PFGE), plasmid profiles, specific primers and probes for PCR and nucleic acid hybridization or 16S rRNA sequencing and sequence comparison for the identification and typing of the bacterial isolates has enhanced the identification of culturable components.

Benno and Mitsuoka[1] indicated that in infants the predominant fecal organisms are bifidobacteria followed by bacteroides, clostridia, enterobacteria, and streptococci, with *Bacteroides* being the predominant genus in adults accompanied by a regular appearance

of *Bifidobacterium*.[5] Benno and Mitsuoka[1] also studied the development of intestinal microbiota from infancy to adulthood and beyond. Comparisons were made with intestinal microbiota analysis of several animal species. The fecal microbiota of adult humans is stable in composition when considered in terms of total population of obligate anaerobes (*Bifidobacterium* and *Bacteriodes* strains), but there is variability in the strain composition.[6]

In general, the bifidobacterial microbiota of adults consisted mainly of strains of *Bifidobacterium adolescentis*; other species detected include *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobaterim catenulatum*, and *Bifidobacterium pseudocatenulatum*.[5,7] In elderly people, *B. longum* seems to predominate, followed by *B. adolescentis* and *B. bifidum*.[8]

Morelli et al.[9] reported that only a few strains of *Lactobacillus* are permanent or autochthonous strains, with great variability between individuals. *L. reuteri*, *L. gasseri*, and, in some cases, *L. salivarius* have been considered have the authentic autochthonous lactobacilli by some authors,[5] whereas others have pointed to *L. acidophilus* and *L. crispatus*.[6]

Bacteroides distasonis, *Bacteroides vulgatus*, and *Bacteriodes fragilis* are the *Bacteriodes* species most commonly found in infants, whereas in adults *Bacteriodes thetaiotaomicron* predominates.[10]

2. Culture in Nonselective Media Followed by Specific Counting or Identification

In this group could be placed techniques that do not require the use of selective media because the selective counting or identification of colonies is carried out once the microorganisms have grown and formed colonies on the plate. For example, monoclonal antibodies have been developed for selective enumeration of *Bacteroides vulgatus* and *Bacteroides distasonis* on fecal samples after dilution and plating in a nonselective medium, avoiding the use of selective agents to which part of the *Bacteriodes* population could be sensitive.[11] On the other hand, oligonucleotide probes have been used for the identification and quantification of intestinal microbiota by means of colony hybridization.[12,13]

Several different culture-dependent techniques have been used for quantitative and qualitative characterization of human intestinal microbiota, but differences in species composition and quantitative contents can be found between the results obtained by different authors. These differences may be due to the high variability of the intestinal microbiota between individuals as well as the methods used (e.g., different culture media for the counting or isolation of strains, different methodologies used for the isolates identification).

On the other hand, conventional culture-dependent methods have limitations such as low sensitivity, long duration, bias introduced due to the culture, and recovery of only culturable species of the intestinal microbiota. This can lead to the overestimation of some species and underestimation of others. Thus, new more rapid, accurate, and specific methods for the detection and quantification of intestinal bacteria are needed in order to obtain more precise knowledge about human intestinal microbiota.

B. Culture-Independent Techniques

Recent developments in molecular biology have led to the application of alternative methods in addition to traditional culture methods. These culture-independent techniques have been applied for intestinal microbiota assessment. One widely used approach employs 16S rRNA and its encoding genes as target molecules. Specific PCR primers

and probes can be designed based on the variable regions of this molecule to detect certain species or groups of bacteria.

Using comparative analysis of 16S rRNA sequences amplified from human feces, Suau and coworkers[14] showed that less than 25% of the molecular species identified corresponded to known organisms, suggesting that many bacteria in the large intestine have not been described yet and that classical culture-based methods have not provided an accurate representation of this community. On the other hand, the percentages of cultivated microbiota obtained by different authors comparing counts obtained by microscopic observation and culture media range from 10 to 58%,[14,15–17] showing the limitations of the culture-based approaches for the study of complex communities.

1. PCR Primers

There are now several reports on developed species or group-specific primers for the detection of different microorganisms predominant in the gastrointestinal tract.[18–22] 16S rDNA–targeted PCR primers enable rapid and specific detection of a wide range of bacterial species, thus procedures in which these primers are used have widespread use in intestinal microbiota assessment. Using PCR amplification with species-specific primers, Wang et al.[19] analyzed the fecal microbiota, showing that *Fusobacterium prausnitzii*, *Peptostreptococcus productus*, and *Clostridium clostridiiforme* had the highest PCR titers. Matsuki et al.[21] studied the bifidobacteria microbiota in fecal samples and found that in adults *Bifidobacterium catenulatum* and not *B. adolescentis*, as had been previously indicated, is the most commonly found organism, whereas *B. breve*, *B. infantis*, and *B. longum* are the predominant species in infants.

2. Hybridization Probes

Several probes have been developed for the assessment of intestinal microbiota.[12,13,15,23–25] These probes have been used for specific culture-independent detection and quantification of different intestinal microorganisms by means fluorescent in situ hybridization[15,23–25] or dot blot hybridization.[26–28]

3. Polymerase Chain Reaction–Enzyme Linked Immunosorbent Assay (PCR-ELISA)

This technique consists of the combination of PCR amplification with an immobilized specific probe and the use of antibodies for the detection of the hybridized amplified DNA. The amplified DNA is labeled, commonly with digoxigenin, and hybridized with a specific detection probe that is immobilized in microtiter plate wells. The presence of hybridized DNA is determined by using digoxigenin-targeted antibodies. This methodology has not been used in many studies, but has been used for the analysis of *Bifidobacterium* species composition in human feces during a feeding trial.[29]

4. Sequence Analysis of Randomly Amplified 16S RNA Genes

Another procedure that has been used in intestinal microbiota research is PCR amplification of 16S rRNA genes in a sample using universal or group-specific primers followed by cloning and sequencing of the amplified DNA. By means of sequencing analysis of 16S rDNA amplicons obtained with *Lactobacillus* group-specific primers, Heilig and coworkers[30] showed that *Lactobacillus ruminis* is the predominant *Lactobacillus* species in fecal samples.

Using primers targeted for the domain Bacteria, Suau and coworkers[14] found that the predominantly cloned sequences in fecal samples belonged to *Bacteroides* and

Clostridium. Surprisingly, no sequences belonging to the genus *Bifidobacterium* were detected, probably indicating some problems during the amplification. The number of PCR cycles can significantly distort the representation of some organisms in the ecosystem due to preferential amplification of some rDNA.[16]

5. TGGE-DGGE Analyses of 16S rRNA Genes

These techniques, widely used for intestinal microbiota assessment, consist of PCR amplification of 16S rRNA genes with group or specific primer pairs, one of which has a GC clamp attached to the 5′ end in order to avoid complete dissociation of the two DNA strands of the amplified product even in stringent denaturing conditions, followed by denaturing gel electrophoresis (TGGE or DGGE) in which the double-stranded DNA is electrophoresed through a gradient of denaturant. The position in a gel where the double-stranded DNA melts and becomes single-stranded DNA depends on the sequence, so amplified DNA will migrate in a sequence-dependent manner. This method is a powerful tool for monitoring bacterial succession phenomena. In addition, the predominant bands obtained can be sequenced in order to determine which organisms are predominant. Using TGGE, Zoetendal et al.[31] studied the diversity of predominant bacteria in human adult fecal samples, showing remarkable stability in the profiles during time. *Eubacterium hallii*, *Ruminococcus obeum* and *Fusobacterium prausnitzii* were the most commonly found species in human feces.

Satokari and collaborators[32] studied the bifidobacteria microbiota in adults using DGGE, showing that *B. adolescentis* is the most commonly found species in adult human feces. With regard to the *Lactobacillus* population, *L. ruminis* and *L. salivarius* have been reported to be the true autochthonous lactobacilli, whereas other species frequently used in food manufacture can be detected in feces of individuals for a long time.[33]

6. Terminal Restriction Fragment Patterns Analysis

Another technique used in intestinal microbiota research is 16S rDNA terminal restriction fragment pattern (TRFP) analysis. 16S rDNA amplification is accomplished with a fluorescently labeled and an unlabeled primer so that the PCR product is labeled at one end. After digestion of the PCR products with one or more endonucleases, the length of the labeled terminal restriction fragments is determined by capillary electrophoresis. Rapid identification of the predominant human and animal intestinal bacteria can be accomplished with this method using the appropriate restriction enzymes.[34,35]

7. Oligonucleotide Arrays

Wang and coworkers[36] developed an oligonucleotide microarray using species-specific probes for the detection of 20 predominant human intestinal bacteria from fecal samples. Microarray technology can be used for simultaneous detection of thousands of target DNA sequences at one time. Thus, its use could permit the detection of thousands of bacterial species in a sample in a rapid and accurate manner. However, in order to avoid the necessity of this expensive microarray equipment, a membrane-array procedure has also been reported.[37] In the near future with the increasing availability of genome sequences from intestinal bacteria, microarray analysis will become a powerful and valuable tool to assess the microbial composition of the human intestinal tract and to study how different members of the intestinal microbiota modulate the expression of genes from both intestinal cells and other intestinal bacteria.

These culture-independent approaches for assessment of intestinal microbiota have led to a better understanding of the qualitative content and the predominant species of the intestinal microbiota, but unfortunately they do not provide reliable quantitative data or information about minority species also present in the gastrointestinal tract, thus some quantitative culture-independent methods have been developed. The procedure most widely used is fluorescence in situ hybridization with fluorescently labeled probes. Recently, other quantitative procedures have been introduced, e.g., real-time PCR.

8. Fluorescent in situ Hybridization Technique (FISH)

Using FISH with different group-specific probes, around 90% of the total fecal bacteria can be detected. *Bacteriodes/Prevotella* and *Clostridium coccoides/Eubacterium rectale* are present in the highest numbers (10^{10}).[23,25] Other bacterial groups represented in high levels ($>10^9$ cells/g feces) are *Ruminococcus* [25] and *Bifidobacterium*.[15,23,25]

FISH has also been used to assess changes in levels of the predominant groups of intestinal bacteria as a result of the consumption of prebiotic substances[38] or probiotic bacteria.[39] By means of FISH it has also been shown that there are some differences in the gut microbiota between infants who later develop atopy and those who do not.[40]

FISH has been relatively widely used for intestinal microbiota assessment, and therefore many data exist. This offers an advantage for new studies. However, the technique is laborious, with problems involving the visual counting of samples, and it is quite time consuming. Such factors limit its further applicability. Alternative methods have been developed to replace manual visual counting with more efficient and objective methods, such as automated image analysis[41] or flow cytometry.[42]

In FISH differences exist in target region availability, cell permeability, or when using 16S rRNA–targeted probes to the ribosome content of the cells. Low fluorescence levels in positively hybridized cells can also significantly overlap high signals of the negative controls.[15] Coaggregation of bacteria, broken cells, or contaminating substances can make counting even more difficult. Therefore, a more rapid and accurate procedure for quantitative microbiota assessment is needed for the future.

9. Real-Time PCR

Real-time PCR (Fig. 1) is a promising tool to study the composition of complex communities such as are found in the gastrointestinal tract. This procedure has been used for the quantification of *Bifidobacterium* in fecal samples[43] and *Escherichia coli* and *Bacteroides vulgatus* in gastrointestinal mucosa.[44] It has also been used for the quantification of total bacteria and some specific species in dental plaque and caries dentine.[45,46] Mainly 16S rDNA genes are used as target molecules, but as more bacterial sequences become available, new specific primers and probes targeting other genes will be available to be used in cases in which the 16S rDNA is not an adequate target. Bacterial quantification by real-time PCR can be influenced by differences in the number of rRNA operons between the quantified species or groups, sequence heterogeneity between different operons in the same species, or differential amplification of different DNA molecules.[47,48]

Another method used for assessing intestinal microbiota structure without the need of cell culture is the analysis of cellular fatty acid profiles in fecal samples.[27] Also, metabolic activities can be used as a crude signature of the microbiota. Compositional changes can be tracked by noting changes in metabolic activities assignable to the microbiota.[49]

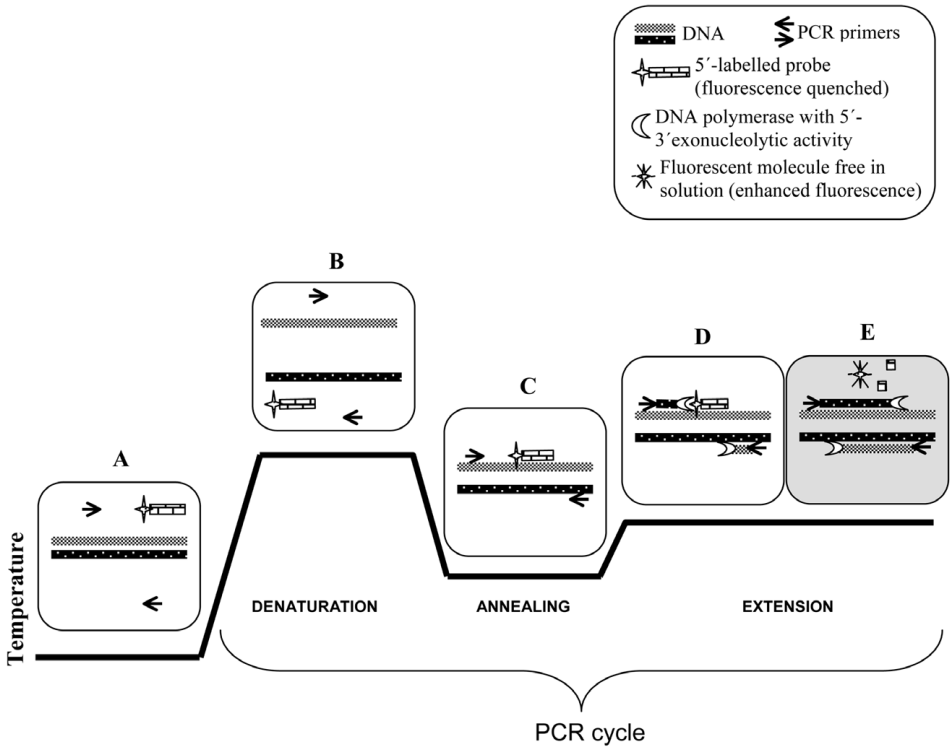

Figure 1 5′exonuclease real-time PCR principle. (**A**) Double-stranded DNA, primers and 5′-labeled probe (fluorescence is quenched) free. (**B**) Denaturation step—double-stranded DNA is denatured. (**C**) Annealing step—annealing of primers and 5′-labeled probe with their complementary sequences; the probe is situated downstream from one of the primers. Extension step: (**D**) the new DNA strands are extended by the DNA polymerase; (**E**) during the extension the DNA polymerase encounters the hybridized probe and digest it by means of its 5′-3′exonucleolytic activity, the fluorescent molecule is released, and an increase in fluorescence can be detected.

V. CONCLUSION

The intestinal microbiota is a dynamic and complex ecosystem that varies greatly between individuals, making its study and characterization difficult. Numerous methodologies, both culture-dependent and culture-independent, have been used for intestinal microbiota assessment and have led to an increase of our understanding of microbiota-host interactions. In recent years, new molecular techniques have enhanced our understanding of microbial ecology in the gut but at the same time have shown that our knowledge of intestinal microbiota composition is limited. In order to obtain more precise data, new rapid and more accurate techniques for intestinal microbiota assessment are urgently needed. New methodologies should provide valuable assistance to future studies and could lead to a significant increase in our understanding of intestinal microbiota composition and microbiota-host interactions. Real-time PCR is a promising tool for studies of intestinal microbiota composition. The development of DNA microarrays for both intestinal cells and intestinal microbiota members will fundamentally change our understanding of

microbiota-host interactions, which will lead to the development of a new generation of probiotics, whose action will be target and site-specific.

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11

Antimicrobial Components from Lactic Acid Bacteria

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I. INTRODUCTION

The ability of lactic acid bacteria (LAB) to produce antimicrobial substances has historically long been used to preserve foods. Preservation of milk by fermentation has been used early in history, Sumerian writings about dairying go back to about 6000 B.C. Procedures for the fermentation of meat were developed as early as the fifteenth century B.C. in Babylon and China. Methods for the fermentation of vegetables were known in China in the third century B.C.[1] Since the days of Metchnikoff, lactic acid bacteria have also been used as probiotics to improve the composition and activity normal microbiota of the intestine.[2]

Fermentation reduces the amount of available carbohydrates and results in a range of small molecular mass organic molecules that exhibit antimicrobial activity, the most common being lactic, acetic, and propionic acids.[3] In addition to the production of these inhibitory primary metabolites, many other antimicrobial components can be formed by different lactic acid bacteria. One should bear in mind that antimicrobial substances are not produced for human convenience. The biological significance is thought to be that of amensalism, a means of one bacterium gaining advantage over another competing microbe. This can be achieved by changing the environment, e.g., acidification, or production of toxins against competitors.[4,5]

The aim of this chapter is to discuss the formation, spectrum of activity, and mode of action of the various antimicrobial substances produced by lactic acid bacteria.

II. ORGANIC ACIDS

Upon fermentation of hexoses, lactic acid is produced by homofermentation or equimolar amounts of lactic acid, acetic acid/ethanol, and CO_2 are produced by heterofermentation (see Chapter 1).

It has long been observed that weak acids have a more powerful antimicrobial activity at low pH than at neutral pH.[6] Of the two acids, acetic acid is the strongest inhibitor and has a wide range of inhibitory activity, inhibiting yeasts, molds, and bacteria,[3] while propionic acid has been observed to exert a strong antimicrobial effect, in particular towards yeasts and molds.[7] This stronger antimicrobial activity of acetic and propionic acid can be explained in part by their higher pK_a of as compared to lactic acid (4.87, 4.75, and 3.08, respectively). At, for example, pH 4, only 11% of lactic acid is undissociated, whereas 85% of acetic acid and 92% of propionic acid is undissociated.[8] When a mixture of acids is present, it is likely that lactic acid contributes mainly to the reduction in pH, while propionic and acetic acid, which become undissociated, are the actual antimicrobial agents. Indeed, mixtures of lactic and acetic acid have been observed to reduce the growth rate of *Salmonella enterica* ser. var. Typhimurium more than either acid alone, suggesting a synergistic activity.[9] However, in addition to reducing the pH, lactic acid has been observed to also permeabilize membranes, thereby further enhancing the activity of other antimicrobial substances.[10]

It is often assumed that the undissociated molecule is the toxic form of a weak acid, although the dissociated acids have also been observed to inhibit microbial growth.[8] It is assumed that the undissociated (neutral) form of the organic acid diffuses across the cell membrane because they are lipid-soluble.[11,12] However, some workers have suggested an energy-linked uptake of certain acids.[13] After entering the cell, the acid will dissociate since the cytoplasmic pH is usually around neutral.[14,15] Many workers have suggested that the release of protons into the cytoplasm leads to acidification and dissipation of the pH gradient over the membrane causing the observe growth inhibition.[3,16] However, other workers suggest that this hypothesis should be reviewed. It has been suggested that not the proton translocation,[17–19] but the accumulation of the anion is the major cause of the observed growth inhibition. It is proposed that the anion reduces the rate of macromolecule synthesis[13] and affects transport over the cell membrane.[17] In particular, lactic acid bacteria, as well as other bacteria, counteract the effects of anion accumulation by reducing their cytoplasmic pH.[19]

In most practical applications, the organic acids will be present in a medium containing organic material. Gelinas and Goulet[20] observed that organic matter reduced the bactericidal activity of a sulfated oleic acid. Cherrington and coworkers[13] found that yeast extract reduced the activity of lactic and acetic acid whereas blood and milk enhanced the activity of acetic acid. We have also observed that the addition of casamino acids (10 g/L) improved the antimicrobial activity of *Lactobacillus fermentum* spent culture liquid.[21]

III. HYDROGEN PEROXIDE

In the presence of oxygen, lactic acid bacteria are able to generate hydrogen peroxide (H_2O_2) through the action of flavoprotein-containing oxidases, NADH oxidases, and superoxide dismutase. In the absence of a source of heme, lactic acid bacteria will not produce catalase for the removal of hydrogen peroxide. Other systems that eliminate hydrogen peroxide are less active than the ones producing it. This allows for the accumulation of hydrogen peroxide.[22] However, Fontaine and coworkers[23] argue that hydrogen peroxide does not accumulate to significant amounts in vivo, because it is decomposed by peroxidases, flavoproteins, and pseudocatalase. The bactericidal effect of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cell; sulfhydryl

groups of cell proteins and membrane lipids can be oxidized.[24–26] Also, some of the hydrogen peroxide–producing reactions scavenge oxygen, thereby creating an anaerobic environment that is unfavorable for certain organisms. It has been suggested that hydrogen peroxide production is particularly important for colonization of the urogenital tract by lactobacilli. Colonization by such lactobacilli has been found to decrease the acquisition of human immune deficiency virus (HIV) infection, gonorrhea, and urinary tract infections.[27] Nevertheless, some workers question whether in vivo hydrogen peroxide per se has significant bactericidal activity.[23,28]

Under natural conditions, the antimicrobial effects of hydrogen peroxide may be enhanced because of the presence of lactoperoxidase and thiocyanate (SCN^-). The glycoprotein lactoperoxidase is found in saliva, tears, and milk. It catalyzes the oxidation of thiocyanate by hydrogen peroxide, generating hypothyanite ($OSCN^-$) and, in the presence of an excess of hydrogen peroxide, also O_2SCN^- and O_3SCN^-:[22,29]

$$SCN^- + H_2O_2 \xrightarrow{\text{lactoperoxidase}} OSCN^- + H_2O$$

Structural damage and changes in bacterial membranes due to exposure to $OSCN^-$ have been reported.[30] However, the main antimicrobial effect is contributed to blocking of the glycolysis. It is proposed that it inhibits glucose transport, hexokinase activity, and glyceraldehyde-3-phosphate dehydrogenase activity due to the oxidation of sulfhydryle groups in these metabolic enzymes. The latter enzyme, glyceraldehyde-3-phosphate dehydrogenase, appears to be the primary target.[31] The activity toward gram-positive bacteria, including lactic acid bacteria, is generally bacteriostatic, whereas many gram-negative bacteria are rapidly killed.[3,22,26]

IV. CARBON DIOXIDE

Carbon dioxide (CO_2) is mainly formed during heterofermentative lactic acid fermentation of hexoses, but also many other metabolic pathways generate carbon dioxide during fermentation.[32] Carbon dioxide has a dual antimicrobial effect. Its formation creates an anaerobic environment and carbon dioxide in itself has an antimicrobial activity.[26] The mechanism of this activity is unknown, but it has been suggested that enzymatic decarboxylations are inhibited[33] and that accumulation of carbon dioxide in the lipid bilayer causes dysfunction in membrane permeability.[26] At low concentrations carbon dioxide can stimulate the growth of some organisms, whereas at higher concentrations it can prevent growth.[26]

Because of its antimicrobial activity, carbon dioxide is now commonly used as the main component of modified atmosphere packages. Gram-negative bacteria have been reported to be more sensitive to the carbon dioxide in the modified atmosphere than gram-positive bacteria.[34]

V. DIACETYL

Diacetyl (2,3-butanedione) was identified by van Niel and coworkers[35] as the aroma and flavor component in butter. In 1927 Lemoigne[36] described its antimicrobial activity against *Bacillus* sp. It is produced by species and strains of the genera *Lactobacillus, Leuconostoc, Pediococcus*, and *Streptococcus*, as well as other organisms.[37] When

hexoses are metabolized, the formation of diacetyl will be repressed. However, diacetyl can be overproduced if citrate is metabolized. Citrate is converted via pyruvate into diacetyl.[26]

Jay[37] observed that diacetyl was progressively more effective at pH <7. It was also observed that the antimicrobial activity was antagonized by the presence of glucose, acetate, and Tween 80. Diacetyl was found to be more active against gram-negative bacteria, yeasts, and molds than against gram-positive bacteria; lactic acid bacteria were the least sensitive. Diacetyl is thought to react with the arginine-binding protein of gram-negative bacteria and thereby interfering with the utilization of this amino acid.[37]

VI. LOW MOLECULAR WEIGHT ANTIMICROBIAL SUBSTANCES

There are several reports on the production of low molecular weight components with antimicrobial activity by lactic acid bacteria.[38–43] In addition to a low molecular weight, these components also share other properties: (a) active at low pH, (b) thermostable, (c) broad spectrum of activity, and (d) soluble in acetone.[44] Detailed information on the substances has not been published. In some cases, other investigators have not been able to reproduce the findings.[45] The question is therefore whether the observed activities are attributable to the reported substances or if they are caused by the previously mentioned metabolic end products. This illustrates the uncertainties surrounding this class of antimicrobial substances.

In the following sections, three well-identified low molecular weight antimicrobial substances will be discussed.

A. Reuterin

Lactobacillus reuteri is a member of the normal microbiota of the gastrointestinal tract of humans and many other animals. It has been found to produce a low molecular weight antimicrobial substance, reuterin, when grown anaerobically on a mixture of glucose and glycerol or glyceraldehyde.[46] Through dehydration of glycerol, *Lb. reuteri* can produce 3-hydroxypropanal, reuterin (Fig. 1). This can subsequently be reduced to 1,3-propanediol by NADH + H^+-dehydrogenase.[3,47] During log phase, no reuterin is produced since it is reduced by the reducing power from glucose metabolism. However, when cells enter stationary phase, reuterin starts to accumulate.[44] When allowed to be in contact with the target cells, *Lb. reuteri* is stimulated in its reuterin production.[48] In aqueous solutions, reuterin can be present in three forms: mainly monomeric, hydrated monomeric, and to a lesser extend cyclic dimeric. It is not known which form or combination is more biologically active.[49] Although other bacteria also dissimilate glycerol via the same

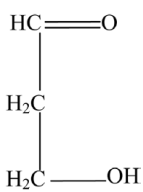

Figure 1 Structure of reuterin, 3-hydroxypropanal.

pathway, accumulation and excretion of reuterin appears to be a specific property of *Lb. reuteri*.[47,50] Reuterin has a very broad spectrum of antimicrobial activity. It was found to have antibacterial, antifungal, antiprotozoal, and antiviral activity.[46,48,51] No reports on possible negative effects of reuterin on human cells could be found, although reuterin has been observed to be able to cross link biological tissues in a similar way to glutaraldehyde.[52] Lactic acid bacteria, including *Lb. reuteri*, are also sensitive to reuterin, but they are more resistant than other microorganisms.[48,51]

The broad antimicrobial activity may be explained by its mechanism of action. Reuterin is thought to act against sulfhydryl enzymes. It was shown to be an inhibitor of the substrate-binding subunit of ribonucleotide reductase, thereby interfering with DNA synthesis.[51]

B. Reutericyclin

A strain of *Lactobacillus reuteri* has recently been observed to produce yet another low molecular weight antimicrobial substance, reutericyclin. Reutericyclin has a molecular weight of 349 Da and is negatively charged and highly hydrophobic.[53] The presence of fatty acids in the culture medium was found to affect its production and the distribution of reutericyclin between the culture supernatant and producer cells. Tween 80 stimulated production most and also significantly increased the concentration of reutericyclin in the culture supernatant. The inhibitory activity of reutericyclin is increased dramatically at higher salt concentrations (2%) and low pH (4.5). The minimal inhibitory concentration was found to be approximately 0.05–1 mg/L for gram-positive bacteria. Gram-negative bacteria and yeasts were not found to be sensitive (>100 mg/L).[54] Reutericyclin does not appear to form pores in the membrane of the target cells, but rather works as a proton ionophore. It partitions into the cytoplasmic membrane, due to its hydrophobicity, and selectively dissipates the transmembrane ΔpH.[55]

C. 2-Pyrrolidone-5-carboxylic Acid

Pyroglutamic acid, or PCA (Fig. 2), was found to be produced by *Lactobacillus casei* ssp. *casei*, *L. casei* ssp. *pseudoplantarum* and *Streptococcus bovis*,[56,57] though PCA is also present in fruits, vegetables, and grasses. It was observed to be inhibitory to *Bacillus subtilis, Enterobacter cloacae, Pseudomonas putida*, and *Pseudomonas fluorescens.* PCA was heat stable (20 min at 121°C) but lost its activity when the pH was raised above 2.5, depending on the target strain used. Although PCA had a stronger antimicrobial activity than lactic acid at the same concentration,[58] it is likely that PCA's mechanism of action is similar to that of organic acids.

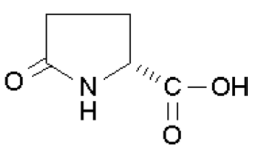

Figure 2 Structure of 2-pyrrolidone-5-carboxylic acid (PCA).

VII. BACTERIOCINS

Bacteriocins are ribosomally synthesized compounds produced by bacteria in order to inhibit the growth of other bacteria. These compounds are found in almost every bacterium species examined to date,[59] but only some of them have been extensively studied. Bacteriocins can be regarded as antibiotics, but they differ from antibiotics in several critical ways: (a) bacteriocins are ribosomally synthesized, (b) host cells are immune to them, (c) their mode of action is different from antibiotics, and (d) they have a narrow killing spectrum and thus they are generally able to kill only bacteria closely related to the producing strain.[60] So far there is not enough evidence that bacteriocins produced by gram-positive bacteria have any effect on gram-negative bacteria without addition of any membrane-active compound. This has limited the use of bacteriocins in food applications, but this property can also be considered an advantage. Bacteriocins can be used as "target drugs" toward specific pathogens without disturbing the beneficial microbiota. Bacteriocins could then limit the use of antibiotics to some extent and thus delay the evolution of antibiotic resistance. Besides concern about antibiotic resistance, increasing consumer awareness of potential health risks associated with chemical preservatives has increased interest in bacteriocins. Bacteriocins are naturally produced so they are more easily accepted by consumers. This section is an introduction to the field of bacteriocins. Many excellent reviews on this topic have been published, and the reader is referred to these for more in-depth information.[59,61–67]

Bacteriocins produced by lactic acid bacteria can be divided into three major classes (Table 1): I, lantibiotics; II, small heat-stable peptides; and III, large heat-labile proteins.[68] A fourth class of bacteriocins with a complex structure has also been suggested, but is not generally accepted. Classes I and II are the main classes of bacteriocins due to their abundance and potential use in commercial applications. Although knowledge about

Table 1 Classes of Bacteriocins Produced by LAB

Class	Subclass	Description
Class I (lantibiotics)	A(1)	Elongated, cationic, membrane active, slight + or − net charge
	A(2)	Elongated, cationic, membrane active, highly − net charge
	B	Globular, inhibit enzyme activity
Class II		Small (<10 kDa), moderate (100°C) to high (121°C) heat-stable, non–lanthionine-containing membrane-active peptides
	IIa	*Listeria* active peptides with –Y–G–N–G–V–X–C– near the amino terminus
	IIb	Two-peptide bacteriocins
	IIc	Other peptide bacteriocins
Class III		Large (>30 kDa) heat-labile proteins
Class IV		Complex bacteriocins: protein with lipid and/or carbohydrate

Source: Adapted from Ref. 114.

bacteriocins has increased greatly, there are still many open questions regarding immunity (self-protection) and the molecular basis of target-cell specificity. The following sections will deal with the four classes of bacteriocins in greater detail, including examples of bacteriocins in each class (producer strain, spectrum of activity), their molecular mechanisms of activity, and protection against it (immunity).

A. Class I

Class I bacteriocins, also called lantibiotics, are small (<5 kDa) peptides. Lantibiotics contain unusual amino acids not normally found in nature (e.g., lanthionine and β-methyllanthionine), in addition to a number of dehydrated amino acids.[69] These unusual amino acids are synthesized by posttranslational modifications. Lantibiotics can be divided into two groups on the basis of their structural and functional features (Table 2). Type A lantibiotics are elongated and cationic peptides up to 34 residues in length. These peptides

Table 2 Examples of Class I Bacteriocins (Lantibiotics) Produced by LAB

Lantibiotic	Producing strain(s)	Antimicrobial activity
Type A lantibiotics[a]		
Type A(I)		
Mutacin B-Ny266	*Streptococcus mutans* Ny266	
Mutacin 1140	*S. mutans* JH1000	
Nisin A	*Lactococcus lactis* several strains, e.g., NIZOR5, 6F3, NCFB894, ATCC11454	*Lactococcus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Micrococcus* spp., *Pediococcus* spp., *Lactobacillus* spp., *Listeria* spp., *Mycobacterium* spp., *Clostridium* spp. (+ spores), and *Bacillus* spp. (+ spores)
Nisin Z	*Lc. lactis*, several strains, e.g., N8, NIZO22186	As above
Type A(II)		
Carnocin U149	*Carnobacterium piscicola* U149	*Carnobacterium* spp., *Lactobacillus* spp., *Pediococcus* spp., and *Lactococcus* spp.
Cytolysin	*Enterococcus faecalis* DS16	
Lacticin 481	*Lc. lactis* CNRZ481, ADRIA85LO30	Lactic acid bacteria and *Clostridium tyrobutyricum*
Lactocin S	*Lactobacillus sake* L45	*Lactobacillus* spp., *Pediococcus* spp., and *Leuconostoc* spp.
Salivaricin A	*S. salivarius* 20P3	*Micrococcus luteus*
Streptococcin A-FF22	*S. pyogenes* FF22	
Type-B Lantibiotics	Not produced by LAB	

[a]Separated into two groups based on leader sequences and gene cluster composition.
Source: Refs. 63, 68, 87, 115.

primarily act by disrupting the membrane integrity of target organisms. Type B lantibiotics are globular, up to 19 residues in length, and act through disruption of enzyme function, e.g., by inhibiting the cell wall biosynthesis of the cell.[63] Lantibiotics are synthesized in an inactive form with a N-terminal leader sequence, which is cleaved off during maturation, releasing active peptide. According to de Vos and coworkers, Type A lantibiotics can be classified further into two groups on the basis of size, charge, and sequence of this leader peptide.[70]

The model-type lantibiotic nisin is discussed as an example of lantibiotics. Nisin was discovered in 1928 by Rogers and coworkers, and to date it is the most widely used commercial bacteriocin sold in more than 40 countries.[64,71] Nisin is produced by several strains of *Lactococcus lactis*, and it has a broad spectrum of activity against gram-positive bacteria. Nisin has been shown to be bactericidal to most lactic acid bacteria, *Staphylococcus aureus*, *Listeria monocytogenes*, vegetative cells of *Bacillus* spp., and *Clostridium* spp. as well as preventing outgrowth of spores in *Bacillus* and *Clostridium* species.[68] Nisin has a natural variant, nisin Z, with a single amino acid change where histidine in position 27 is replaced with asparagine.[72] This substitution has little effect on antimicrobial activity, but it increases the solubility of nisin at neutral pH, offering more potential applications.

The cytoplasmic membrane is the primary target for nisin activity. *Escherichia coli* and other gram-negative bacteria are only affected when their outer membranes are sublethally damaged with chelating agents, e.g., EDTA, which binds magnesium ions from the lipopolysaccharide layer of the outer membrane. The mode of action of nisin has been studied intensively, and several mechanisms for its antimicrobial activity have been proposed. Nisin has been shown to inhibit peptidoglycan biosynthesis,[73] leading to the suggestion that nisin interacts with cell wall precursors lipid I and lipid II.[74] Later studies showed that nisin is capable of pore formation.[75,76] Further studies showed the dual functionality of nisin when it was demonstrated that nisin uses lipid II primarily as a docking molecule for pore formation.[77] Pores in the membrane allow diffusion of small compounds such as amino acids and ATP, resulting in the collapse of the proton-motive force (PMF), and stop the biosynthesis of macromolecules such as DNA, RNA, and proteins. In addition to inhibition of cell wall biosynthesis and formation of pores, nisin has been shown to be capable of inducing autolysis of susceptible staphylococcal cells. Cationic nisin is able to release two strongly cationic cell wall–hydrolyzing enzymes (*N*-acetylmuramoyl-L-alanine amidase and *N*-acetylglucosaminidase) by a cation exchange-like process, resulting in enzyme activation and lysis of the cells.[65]

Gram-positive bacteriocins, especially lantibiotics, differ from gram-negative ones in that they require many genes for their production. The nisin gene cluster has been identified to contain genes for prepeptide (*nisA*), enzymes for modifying amino acids (*nisB, nisC*), cleavage of the leader peptide (*nisP*), secretion (*nisT*), immunity (*nisI, nisFEG*), and regulation of expression (*nisR, nisK*).[59]

The bacteriocin-producing strain needs to protect itself from the antimicrobial activity of its bacteriocin. Also, target strains can develop resistance to bacteriocin. Klaenhammer defined three categories of nisin resistance: (a) immunity, (b) resistance not genetically linked to production, and (c) nisin-resistance mutation.[68]

1. Nisin immunity is genetically linked with genes *nisI* and *nisFEG.* NisI is an immunity lipoprotein, which is anchored on the outside of the cell membrane.[68] NisFEG is an ATP transporter, which transports nisin out of the

cell, lowering the concentration of nisin below an inhibitory threshold.[78] Full immunity seems to require both gene products, and NisI and NisFEG seem to cooperate in immunity.[79]

2. Many non–nisin-producing gram-positive strains have been found to have nisin-inactivating enzymes or nisinases. *Bacillus cereus* has been found to inactivate nisin by reducing the dehydroamino acids.[80,81]
3. Nisin resistance may be acquired in the presence of sublethal nisin concentrations. The mechanism of resistance may differ from strain to strain.[82] In general, bacteriocin resistance appears to be a complex phenomenon, involving various changes in the bacteria. Klaenhammer suggested mutational changes in cell components directly or indirectly involved with nisin adsorption or membrane insertion.[68] In the case of *L. monocytogenes*, nisin resistance has been ascribed to alterations in both the fatty acid composition and the phospholipid composition.[62]

B. Class II

Class II bacteriocins are also small (<10 kDa), and generally this class consist of heat-stable, non–lanthionine-containing, membrane-active peptides. Typical for this class is that their inhibition spectrum is mostly narrow. This has raised the question whether class II bacteriocins act through a receptor molecule in the target cell membrane. However, the matter of a receptor molecule or molecules is still an unresolved issue. Class II bacteriocins can be divided into three subclasses (Table 3). Class IIa is the largest group, and typical of this subclass is a conserved amino-terminal sequence (YGNGVXC) and activity against *Listeria.* Subclass IIb includes bacteriocins with two peptides. Formerly class IIc consisted of bacteriocins that were thiol-activated and secreted in sec-dependent manner. However, now it has been shown that bacteriocins formerly grouped into class IIc can act with their cysteine residues being oxidized can use the sec-dependent secretion system.[62] Class IIc has therefore been modified to contain other non-lantibiotic bacteriocins or miscellaneous peptides that do not belong to class IIa or IIb.[65,66]

Most class II bacteriocins dissipate the PMF of the target cell. Recent findings have shown that some class II bacteriocins probably need a target molecule at the surface of the sensitive cell. There is evidence that mannose permease of the phosphotransferase system (PTS) could be the target molecule for the subclass IIa bacteriocins mesentericin Y105 and leucocin A.[65] Subclass IIb dissipates PMF, and it seems that lactococcin G likely needs a specific receptor molecule.[65] As subclass IIc is a heterogeneous group of bacteriocins, their modes of action differ. For example, the class IIc bacteriocin Lactococcin 972 is able to inhibit septum formation.[83]

Class IIa bacteriocins are promising for industrial applications because of their strong antilisterial activity. They are even more interesting as antilisterial agents than class I bacteriocins such as nisin, because they do not have a broad inhibitory spectrum and thus do not kill starter cultures.[67] Class IIa bacteriocins have also been called pediocin-like bacteriocins. We use pediocin PA-1 as an example of class II bacteriocins because it is one of the most extensively studied bacteriocins from this class.

Pediocin PA-1 (formerly named also pediocin AcH) is usually produced by *Pediococcus acidilactici.* Pediococci are usually used in the production of fermented vegetables and meat, and they are not well adapted to growth in dairy products. This means that pediocin PA-1 should be used as a pure compound in dairy products. Pediocin PA-1 is

Table 3 Examples of Class II Bacteriocins Produced by Lactic Acid Bacteria (LAB)

Bacteriocin	Producing strain(s)	Antimicrobial activity
Class IIa		
Bavaricin A	*Lactobacillus sake* MI401	*Listeria* spp.
Bavaricin MN	*Lb. sake* MN	*Listeria* spp.
Carnobacteriocin B2	*Carnobacterium piscicola* LV17B	*Listeria* spp., *Enterococcus* spp., *Carnobacterium* spp., *Lb. plantarum*, and *Pediococcus parvulus*
Carnobacteriocin BM1	*Cb. piscicola* LV17B	As above
Curvacin A	*Lb. curvatus* LTH1174	*L. monocytogenes*, *L. ivanovii*, *Lb. curvatus*, *Lb. sake*, *Lb. fructivorans*, *Carnobacterium* spp., and *E. faecalis*
Divercin V41	*Cb. divergens* V41	*Listeria* spp.
Enterocin A	*Enterococcus faecium* CTC492/T136	*Listeria* spp.
Enterocin P	*E. faecium* P13	*L. monocytogenes*, *Clostridium perfringens*, *C. botulinum*, and *Staphylococcus aureus*
Leucocin A/B-Ta11a	*Leuconostoc gelidum* UAL187 *Ln. carnosum* Ta11a	*Listeria* spp.
Mesentericin Y105	*Ln. mesenteroides* Y105	*Listeria* spp.
Mundticin	*E. mundtii* ATO6	*Listeria* spp.
Pediocin PA-1/AcH/ SJ-1	*Pediococcus parvulus* ATO34/ATO77 *P. acidilactici* H/SJ-1/PAC 1.0 *Lb. plantarum* WHE92	"Wide," *L. monocytogenes*
Piscicolin 126	*Cb. piscicola* JG126	*Listeria* spp.
Sakacin 674	*Lb. sake* LB764	*Listeria* spp.
Sakacin A	*Lb. sake* LB706	*L. monocytogenes*, *Cb. piscicola*, *Enterococcus* spp., *Lb. sake*, *Lb. curvatus*, *Lb. brevis*, *Lc. lactis* ssp. *cremoris*, *Ln. paramesenteroides*, *Aeromonas hydrophilia*, and *S. aureus*
Sakacin P	*Lb. sake* LB673	*Listeria ivanovii, Lb. curvatus*, *Lb. delbrueckii*, *Lb. sake*, *Lb. pentosus*, *Lb. plantarum*, *Lb. reuteri*, *Lb. fructivorans*, *Carnobacterium* spp., and *E. faecalis*
Class IIb		
Acidocin J1132	*Lb. acidophilus* JCM 1132	*Lb. acidophilus*

(*continued*)

Table 3 *Continued*

Bacteriocin	Producing strain(s)	Antimicrobial activity
Lactacin F	*Lb. johnsonii* 11088	*Lb. fermentum*, *E. faecalis*, *Lb. delbrueckii*, *Lb. helveticus*, *A. hydrophilia* (?), and *S. aureus* (?)
Lacticin 3147	*Lc. lactis* DPC3147	*Clostridium* spp., *Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., and *S. aureus*
Lactobin A	*Lb. amylovorus* LMG P-13139	"Narrow"
Lactococcin G	*Lc. lactis* LMG2081	Lactic acid bacteria and *Clostridium* spp.
Plantaricin EF	*Lb. plantarum* C11	
Plantaricin JK	*Lb. plantarum* C11	
Plantaricin S	*Lb. plantarum*	*Leuconostoc* spp., *Lb. helveticus*, *Lb. plantarum*, *Lb. delbrueckii*, *Lb. reuteri*, *Enterococcus* spp., *Pediococcus* spp., *Lactococcus* spp., *Streptococcus* spp., *Micrococcus* spp., *Propionibacterium* spp., and *Clostridium tyrobutyricum*
Thermophilin T	*Streptococcus thermophilus*	Lactic acid bacteria, *Clostridium sporogenes*, and *C. tyrobutyricum*
Class IIc		
Divergicin A	*Cb. divergens* LV13	"Narrow"
Lactococcin A	*Lc. lactis*	"Narrow" (*Lactococcus*)
Lactococcin 972	*Lc. Lactis* IPLA972	*Lactococcus* spp.
Plantaricin A	*Lb. plantarum* C-11	*Lb. plantarum*, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Lc. lactis*. and *E. faecalis*

Source: Refs. 62, 65, 66, 87, 89, 90, 116–122.

encoded by an operon containing four genes: structural gene (*pedA*), immunity gene (*pedB*), and secretion genes (*pedC* and *pedD*).[84] Pediocin PA-1, like other low molecular mass bacteriocins, is synthesized as a precursor with a N-terminal leader sequence. Transmembrane translocation of the bacteriocin-peptide is mediated by two membrane-bound proteins: an ABC transporter and an accessory protein.[62] Data show that the N-terminal part of the ABC-transporter is involved in proteolytic removal of the leader sequence from pediocin PA-1.[85]

Many reports about the mode of action of class IIa bacteriocins have been published. How these bacteriocins interact with the membrane is still unknown. One explanation for the interaction is that positively charged and polar residues of class IIa bacteriocins interact with anionic phospholipids in the membrane, but details of this interaction are unclear.[62] It has been proposed that the consensus motif YGNGVXC in the N-terminus of the bacteriocin is involved in membrane binding as well as a specific target molecule at the cell surface has been proposed.[65] The somewhat less conserved C-terminus of the bacteriocin is hydrophobic and/or amphiphilic and it is though to penetrate into the hydrophobic part of the target membrane thereby mediating membrane leakage.[86] Class IIa bacteriocins, like class I bacteriocins, cause depletion of intracellular ATP. However, in the case of class IIa bacteriocins this does not seem be caused through leakage, due to smaller pore sizes formed by class IIa bacteriocins than by lantibiotics. When class IIa bacteriocins are used, ATP depletion has been thought to result from accelerated consumption of ATP in order to maintain or restore PMF and/or the inability of the cell to produce ATP due to phosphate efflux.[62]

It is not known whether immunity proteins have interactions with bacteriocins or whether they can block the pores. Data show that class IIa bacteriocins can provide partial protection against other class IIa bacteriocins ("cross-immunity"). However, there is high homology between class IIa bacteriocins but low homology between the immunity genes. This has been explained by a new model in which the immunity protein acts indirectly with bacteriocins through a receptor.[62]

C. Class III

Class III bacteriocins are defined as large (>30 kDa) heat-labile proteins. This class may, therefore, include bacteriolytic extracellular enzymes (hemolysins and muramidases) that may mimic the physiological activities of bacteriocins.[87] Class III bacteriocins have so far been isolated only from members of the genus *Lactobacillus*.[68] Not so many bacteriocins have been grouped into this class (Table 4) mainly because of lack of knowledge about these larger bacteriocins.

D. Class IV

This additional proposed class contains complex bacteriocins with lipid or carbohydrate moieties which appear to be necessary for activity. The existence of class IV is not

Table 4 Class III Bacteriocins Produced by LAB

Bacteriocin	Producing strain(s)	Antimicrobial activity
Acidophilucin A	*Lactobacillus acidophilus*	*Lb. delbrueckii* and *Lb. helveticus*
Caseicin	*Lb. casei* B40	*Lb. casei*
Helveticin J	*Lb. helveticus* 481	*Lb. helveticus*, *Lb. delbrueckii* ssp. *bulgaricus* and *lactis*
Helveticin V-1829	*Lb. helveticus* 1829	*Lb. helveticus* and *Lb. delbrueckii* spp. *bulgaricus*
Lactacin A	V. *Lb. delbrueckii*	*Lb. delbrueckii* ssp. *lactis*
Lactacin B	VI. *Lb. delbrueckii*	*Lb. delbrueckii* ssp. *bulgaricus* and *delbrueckii*

Source: Refs. 59, 68.

generally accepted, as it may include regular peptide bacteriocins that have not been properly purified. Klaenhammer[68] urges caution with this class, and information related to this class is very limited. Therefore, we do not discuss this class here.

VIII. METHODS FOR STUDYING ANTIMICROBIAL ACTIVITY

Agar well diffusion assay is probably the most commonly used screening method for detection of antimicrobial activity. In this method an indicator strain is inoculated into the agar or plated on the agar and supernatants of potential bacteriocin producers are put into wells cut out of the agar.[88] Positive results from diffusion assay are detectable as clear zones around the wells after overnight incubation. Indicator strains can also be incubated together with supernatants in liquid form, and the result is measured spectrophotometrically or by enumeration of colony-forming units after incubation. In this assay, as well as in agar diffusion assay, used supernatants should be cell-free and pH should be the same in every sample, including the control sample, in order to inhibit the production or presence of organic acids. Usually supernatants are also treated with catalase in order to get rid of hydrogen peroxide. After assays, effective supernatants are selected, and they are treated with proteases (usually at least two, e.g., trypsin and proteinase K). Bacteriocins should lose their activity after protease treatment. If screening has indicated potential bacteriocins, the bacteriocins should be purified (and identified), because it is evident that one type of bacteria can produce more than one bacteriocin.[89,90]

New rapid screening methods for the detection of antimicrobial activity have been developed. In one method, indicator organisms are exposed to bacteriocins after staining with carboxyfluorescein diacetate.[91] Fluorescence is measured by flow cytometry, and the effect of bacteriocin is seen as a decrease of fluorescence when the fluorescent compound is leaked out from cells. Our group has used bioluminescent indicator strains in screening of antimicrobial activity. Luciferase genes are transformed into indicator strains, and indicator strains start to produce light in reaction[92]

$$FMNH_2 + O_2 + \text{R-CO-H} \longrightarrow FMN + \text{R-COOH} + H_2O + \text{light (490 nm)}$$

When bioluminescence is closely linked into the energy metabolism of bacteria, changes in light production are rapid. This increases the sensitivity of the assay and allows for real-time assessment of antimicrobial activity.

IX. SPECIFIC PURIFICATION METHODS FOR BACTERIOCINS

Purification and isolation of bacteriocins is of prime importance for their identification and for studying their mechanisms of action. In addition to the traditional biochemical methods of protein purification; ammonium sulfate precipitation and various forms of chromatography, alternative precipitation methods have been described: Triton X-114[93] and chloroform.[94] These methods make use of the hydrophobic or amphiphilic nature of bacteriocins. More specific methods for the isolation of bacteriocins have also been described.

By raising the pH of the culture supernatant to about 6, Yang and coworkers[95] induced the adsorption of bacteriocins (nisin, sakacin A, Pediocin PA-1, and Leuconocin Lcm1) to the producer cells. By subsequently reducing the pH to 1.5–2.0, desorption of the bacteriocins was induced and high yields of pure bacteriocin were

obtained. Similar adsortion methods have been described using rice hull ash or silicic acid. Maximal adsorption was obtained by incubation at pH 6–9, depending on the bacteriocin tested, while desorption was induced by lowering the pH to 2.5–3.[96]

X. ADHESION INHIBITORS

Although not antimicrobial per se, substances that inhibit the adhesion to the intestinal mucosa are thought to reduce the ability to colonize the intestine[97] and in that sense affect the survival of the microorganisms in question.

Adhesion to a surface is important to bacteria in most environments. It enables them to colonize environments under conditions where they would otherwise be washed away.[98] Due to secretion of fluids and peristaltic movements, the flow in the small intestine is rather high—1–2 cm/min.[99] Adhesion to the intestinal mucosa is regarded as a prerequisite for colonization of the small intestine. Blocking this adhesion will reduce the chances of survival in the intestine.

It has been observed by many workers that whole *Lactobacillus* spp. and bifidobacteria are able to block the adhesion and invasion of many enteropathogens and uropathogens.[100–105] Few reports are available on substances from lactic acid bacteria that inhibit adhesion.

Chan and coworkers[100,101] observed that not only whole cells, but also cell fragments from *Lactobacillus* spp. and a diphteroid organism were able to inhibit the adhesion of gram-negative uropathogens to uroepithelial cells in suspension. Lipoteichoic acids were found to be responsible for the observed inhibition of adhesion. Adhesiveness of the cell fragments and steric hindrance were the major mechanisms of adhesion inhibition.

Lactobacillus fermentum has been found to release a high molecular weight component into its spent culture liquid that reduces the adhesion in vitro of K88ac expressing *Escherichia coli* by at least 50%.[106] The component was concluded to be a cell wall fragment and composed of glucose, *N*-acetylglucosamine, and galactose.[107] The component was found to be active against all K88 serotypes and SfaII fimbriae. Other *Lactobacillus* spp. from enteric origin also produced a similar substance.[108] The mechanism of activity was proposed to be specific binding to mucus glycoproteins and blocking of the receptor sites by steric hindrance.[107] Welin[109] subsequently showed that the substance also inhibits the adhesion of K88ac-expressing *E. coli* to ileal brush borders and reduced its ability to penetrate through ileal mucus in vitro.

Several lactic acid bacterium strains have been shown to produce biosurfactants: *Streptococcus mitis, Lactobacillus fermentum*, and *Lactobacillus rhamnosus*.[110–112] These surfactants exhibited strong adhesive properties against *Enterococcus faecalis, Streptococcus mutans, Escherichia coli, Stapylococcus epidermidis*, and *Candida albicans* to various substrata.[110,113] One of the biosurfactants, surlactin, produced by *Lb. fermentum* RC14, was identified as a 29 kDa protein with close homology to a collagen-binding protein of *Lb. reuteri* and a surface protein of another *Lb. fermentum* strain.[112] Other biosurfactants have been identified as glycolipids, most likely rhamnolipids.[110] The surfactants could be isolated from the culture broth at mid-exponential or early stationary phase. It is likely that the surfactants coat the substratum and thereby change the physicochemical properties of it. This, in turn, will interference with the adhesion of the target strains, either inhibiting their adhesion or releasing bound cells.

XI. CONCLUSIONS

Due to their fermentative metabolism, lacti acid bacteria produce organic acids, important antimicrobial substances. They are the antimicrobial substances that have been used the longest and most widely and have proven to provide a safe means of preserving foods. Other general antimicrobial substances produced widely by lactic acid bacteria include hydrogen peroxide, carbon dioxide, and diacetyl. A few strains produce specific antimicrobial substances like reuterin and pyroglutamic acid. In addition to these general antimicrobial substances, many strains have been found to produce bacteriocins. These often have a more defined antimicrobial spectrum, ranging from only related strains to a wide variety of gram-positive and gram-negative bacteria. The fact that the ability to produce bacteriocins is so widespread among lactic acid bacteria and that a large amount of their energy is spent on the production, secretion, and immunity of the bacteriocins indicates their ecological significance.

The current classification system of bacteriocins will need revision in the future. Instead of a division based on similarities in activity or size, bacteriocins should be classified according to amino acid sequence homology (in analogy to bacterial taxonomy, which is increasingly based on 16 rRNA homology rather than phenotypic characteristics). Such a classification system will also aid in the understanding of the mechanisms of action.

There is continued interest in bacteriocins from an applied perspective, as they are thought to have a potential as natural preservatives. However, it remains to be determined whether these substances will be functional in foods or feeds and if they will be produced and be functional in situ. Thus, despite advances in this field, much work remains to be done.

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12

Lactic Acid Bacteria as a Tool for Enhancing Food Safety by Removal of Dietary Toxins

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I. INTRODUCTION

There is increasing scientific evidence to suggest that certain dietary components can be used as a preventive tool against a number of health disorders. Conversely, other dietary components, such as toxins present in the food supply, can be a cause of health concern. In the industrialized countries up to 10% of the population may suffer from foodborne diseases annually. The risk of foodborne disease is substantially heightened by biological and chemical contamination in areas where food is produced, processed, and consumed. Population growth and unplanned migration from rural areas results in urban slums, which further increase pollution and consequently the incidence of foodborne diseases.

This chapter focuses on a group of natural toxins, the mycotoxins, which are widespread contaminants of many foods and feeds. Aflatoxins, trichothecenes, ochratoxin A, zearalenone, and α-zearalenol are all members of this group, which contains some of the most potent 12 carcinogens known.

Mycotoxins are produced by naturally occurring fungi that grow on a wide variety of grains and nuts. When these contaminated primary products are consumed by farm animals, contamination of animal products can also occur. Mycotoxins are of great concern because of their detrimental effects on the health of humans and animals. These toxins have both acute and long-term toxicity. Once activated metabolically, many mycotoxins can become carcinogenic, mutagenic, teratogenic, and immunosuppressive in nature.[1] Mycotoxins are also responsible for substantial financial losses[2] in a broad spectrum of rural industries. These losses arise from downgrading or destruction of contaminated crops, cracking of cheeses, and impaired growth and feed efficiency of animals fed contaminated feeds.

Mycotoxin levels can be significantly lowered by well-planned strategies, but usually cannot be completely eliminated. Some strategies reported in the scientific literature are clearly more effective and useful than others, but no commercially satisfactory method is available.[3] Hence, there is a great demand for suitable strategies to detoxify contaminated products and mitigate the impact of existing contamination in foods and feeds.

A very attractive opportunity for intervention to reduce exposure to dietary mycotoxins is presented by the bacterial strains we have identified. Two such strains, *Lactobacillus rhamnosus* strain GG (*Lb. rhamnosus* GG) and *Lactobacillus rhamnosus* strain LC-705 (*Lb. rhamnosus* LC705), have been extensively studied and are shown to have the greatest aflatoxin-removal capacity described to date. These strains of lactic acid bacteria (LAB) are currently used in food products and present a potential cost-effective and commercially viable approach to detoxifying aflatoxin-contaminated products. Information on their ability to remove aflatoxins and other mycotoxins both in vitro and in vivo is an area of rapid development. The application of this technology to industry and the development of functional foods may form important areas of detoxification and decontamination biotechnology in the future.

II. BINDING OF MYCOTOXINS BY LACTIC ACID BACTERIA

Several years ago a number of strains of lactic acid bacteria were selected, on the basis of their acclaimed health benefits and related properties, and tested for their detoxification potential. Some strains proved highly effective in removing aflatoxin B_1 (AFB_1) in model systems. It was found, however, that in a given genus and even within a given species, not all strains were equivalent in terms of toxin binding. Unlike the removal of other dietary mutagens by lactic acid bacteria,[4] the capacity for AFB_1 removal was a characteristic of only specific strains, with efficacy varying markedly.[5–9] The results also indicated that aflatoxins are not removed from solution by bacterial metabolism, but rather are bound to the bacteria. Similar results were reported by Oatley et al.,[10] where bifidobacteria bound from 25% to nearly 60% of the AFB_1 added.

Bacterial concentrations must exceed 10^9 bacteria/mL for effective removal of AFB_1.[6] The total number of AFB_1 molecules that can be bound to a single viable bacterium has been estimated to exceed 10^7.

Besides AFB_1, the binding of other aflatoxins—AFB_2, AFB_{2a}, AFM_1, AFM_2, AFG_1, AFG_2—have also been studied.[11–13] In general, these aflatoxins are not bound as effectively as the more toxic and more commonly found AFB_1. Another mycotoxin, ochratoxin A, was also removed (36–76%) by *Lb. rhamnosus* GG and *Lb. rhamnosus* LC705, but not as effectively as AFB_1 (77–92%).[4]

In another study we have investigated the ability of selected strains of *Lactobacillus* and *Propionibacterium* to remove common *Fusarium* toxins, "trichothecenes," from liquid media.[14] The trichothecenes studied were deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), nivalenol (NIV), fusarenon (FX), diacetoxyscirpenol (DAS), T-2 and HT-2 toxins. The bacteria, *Lb. rhamnosus* GG, *Lb. rhamnosus* LC705, and *P. freudenreichii* ssp. *shermanii* JS were incubated in PBS-buffer containing 20 μg of toxin/mL for one hour at 37°C, and after centrifugation the concentration of the toxins in the supernatant fraction was measured. Both viable and heat-killed forms of *Lb. rhamnosus* GG and *P. freudenreichii* ssp. *shermanii* JS were more efficient than *Lb. rhamnosus* LC705 in removing the toxins from liquid media. *Lb. rhamnosus* GG and *P. freudenreichii* ssp. *shermanii* JS removed four of the seven tested toxins (the percentage of removal varying from 18 to 93%) and *Lb. rhamnosus* LC705 removed two toxins (in 10–64%). While DAS was removed by all three bacteria, 3-AcDON was not removed by any of the bacteria, and HT-2 was not removed by nonviable *Lb. rhamnosus* GG and was only slightly removed by nonviable *Lb. rhamnosus* LC705. Binding is postulated as the mechanism of the removal, since no difference was observed between the ability of viable and heat-killed bacteria to remove trichothecenes and no degradation products of the toxins were detected in the gas chromatography–mass spectrophotometric analysis. It is concluded that significant differences exist in the ability of the bacteria to bind trichothecenes in vitro.

The interaction between another two *Fusarium* mycotoxins, zearalenone (ZEN) and its derivative α-zearalenol (α-ZOL), and two food-grade strains of *Lactobacillus* has also been investigated.[15] The mycotoxins (2 μg/mL) were incubated with either *Lb. rhamnosus* GG or *Lb. rhamnosus* LC705. A considerable proportion (38–46%) of both toxins was recovered from the bacterial pellets. No degradation products were detected in the high-performance liquid chromatograms of either the supernatant of the culturing media or the methanol extract of the pellet. Both heat-killed and acid-killed bacteria were capable of removing the toxins, indicating that binding, not metabolism, is the mechanism by which the toxins are removed from the media. Binding of ZEN or α-ZOL by lyophilized *Lb. rhamnosus* GG and *Lb. rhamnosus* LC705 was a rapid reaction, with approximately 55% of the toxins being bound directly after mixing with the bacteria. As expected, binding was dependent on the bacterial concentration. Co-incubation of ZEN and α-ZOL with the bacteria significantly affected the percentage of toxin bound, indicating that these toxins may share the same binding site.

III. MYCOTOXIN-REMOVAL MECHANISMS

Elucidation of the binding mechanism involved here is of crucial importance for the safe use of this novel detoxification approach in the food and feed processing methods. The broad location of the bacterial binding sites and the types of interactions involved have been identified for AFB_1.[12,16,17] AFB_1 appears to bind to the bacterial surface of both *Lb. rhamnosus* GG and *Lb. rhamnosus* LC705.[17] This was indicated by the accessibility of bound AFB_1 to a polyclonal anti-AFB_1 antibody in an indirect competitive inhibition enzyme-linked immunosorbent assay (ELISA). The recovery of up to 99% of bound AFB_1 from the bacteria by solvent extraction[17] is further evidence for bacterial surface binding. Transmission electron microscopy has shown that exopolysaccharides are present for both strains when optimal binding occurs. Judging by the effects of pronase E, lipase, and *m*-periodate on binding,[12] it is suggested that the binding occurs predominantly with carbohydrate and protein components. The effect of the antihydrophobic agent urea on binding

also suggests that hydrophobic interactions are important.[12] Studies over a range of ionic strength (up to 3), using monovalent (NaCl) and divalent ($CaCl_2$) metal ions, and a range of pH (2.5–8.5), showed no substantial effects on AFB_1 binding,[12] implying that electrostatic interactions and hydrogen bonding do not play a major role.

An understanding of the stability of the complexes formed between bacteria and AFB_1 is also critical for safe implementation of this detoxification approach. The stability of the AFB_1 complexes formed with 12 bacterial strains, in both viable and nonviable (heat- or acid-killed) forms, was assessed by repetitive aqueous extraction.[17] After five extractions a significant amount of AFB_1 remained bound, with nonviable bacteria retaining the highest amount of AFB_1. Autoclaving and sonication did not release any detectable AFB_1. Variation in temperature (4–37°C) and pH (2–10) did not have any significant effect on the amount of AFB_1 released. In all cases binding is of a reversible nature and the stability of the complexes formed depends on the bacterial strain, bacterial treatment, and environmental conditions.

The reported binding method constitutes a potential cost-effective and commercially viable approach to detoxifying mycotoxin-contaminated products by employing lactic acid bacteria strains currently used in food products. The method has been tested both under laboratory conditions[6,8] and in food products.[11,13] We are currently applying this approach to develop an industrial method for removing mycotoxins from contaminated liquid products, such as milk and oils.[11,14,15] This method employs an immobilized form of viable or nonviable bacteria. However, certain mechanistic considerations need to be investigated before this biotechnology is applied in practice (Fig. 1). For example, there is a need to investigate (a) the maximal mycotoxin binding per bacterium, (b) the structural requirements for this binding, and (c) the variation in the extent of mycotoxin binding over a range of conditions.

IV. ANIMAL STUDIES

The above in vitro results led to an investigation of the ability of selected strains to bind AFB_1 in vivo, and to testing whether the strength of binding was sufficient to reduce AFB_1 bioavailability. *Lb. rhamnosus* GG, *Lb. rhamnosus* LC705, and *P. freudenreichii* ssp. *shermanii* JS proved capable of reducing the absorption of AFB_1 from ligated duodenal loops of 1-week old chickens. This was indicated by a reduced content of AFB_1 in the soluble fraction of the luminal fluid, reduced mucosal uptake of AFB_1, and increased content of AFB_1 in the bacterial pellet of the luminal fluid.[18] The finding was significant, as it indicated a potential reduction in the bioavailability of AFB_1 through a reduction in its absorption via the intestinal mucosa. The complex formed between the bacteria and AFB_1 was stable under luminal conditions for a period of 1 hour.

The results suggest that dietary decontamination can be accomplished by the addition of specific nonviable probiotic lactic acid bacteria to animal feeds, enabling the binding of aflatoxin in the gastrointestinal tract and its removal via the feces, without harmful effects on the host animal. Such an approach could easily be adapted for use in poultry and other feeds with potential benefits in terms of the enhanced productivity of noncontaminated animals and the production of noncontaminated food products. It will be of great importance to confirm these in vivo findings by conducting experiments under farm conditions, i.e., by introducing both aflatoxin and bacteria via the normal route of exposure (Fig. 2). Aflatoxin levels in blood and organs that are targets for aflatoxin

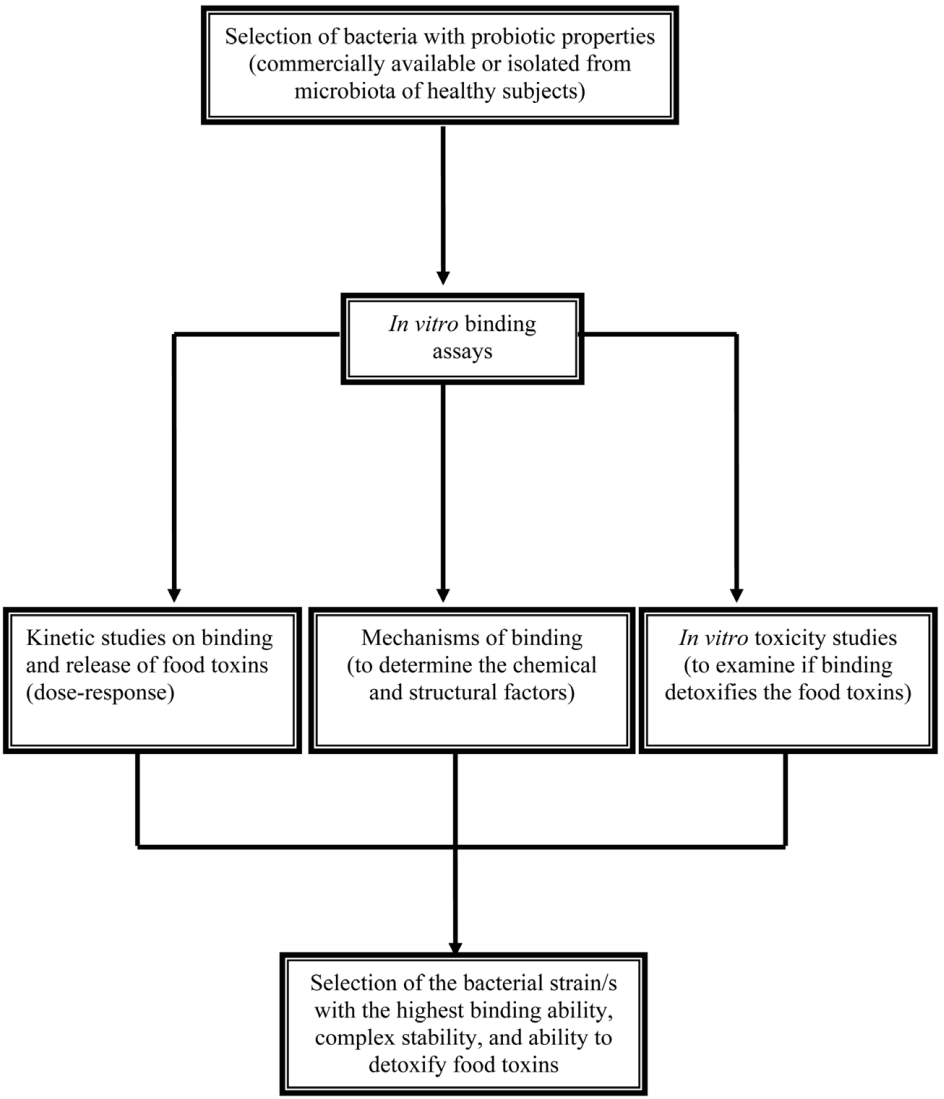

Figure 1 In vitro studies required to identify and explain the interactions between lactic acid bacteria and mycotoxins.

toxicity will need to be ascertained in order to establish the effect of these bacteria on aflatoxin bioavailability.

V. HUMAN STUDIES

A clinical pilot trial was carried out in Egypt to investigate the effect of a probiotic preparation containing both *Lb. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS on the levels of AFB_1 in human fecal samples.[19] Fecal sampling was the only practical sampling method for this field study, and fecal levels of AFB_1 are thought to reflect

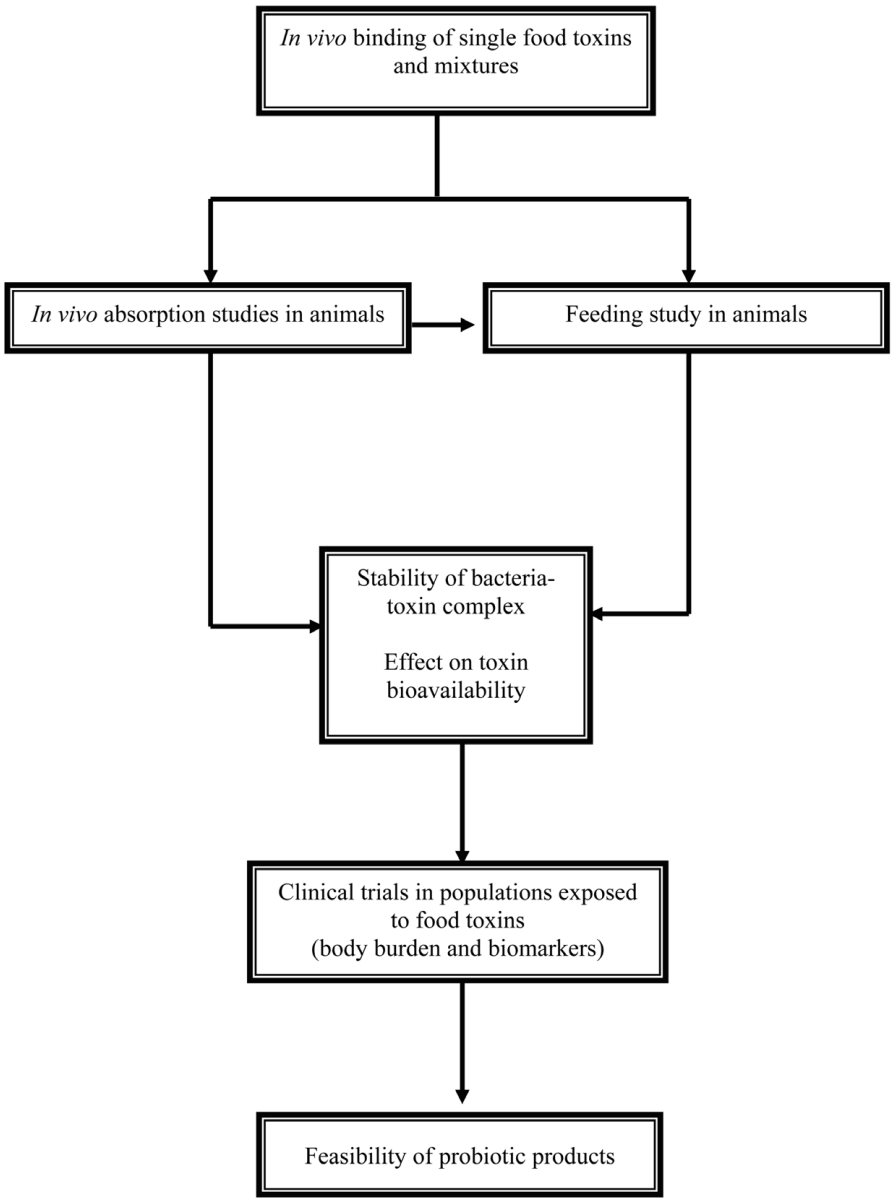

Figure 2 Animal and human studies needed prior to the development of functional food/feed products to be utilized in the reduction or elimination of carcinogenic and toxic effects caused by dietary mycotoxins.

exposure. Twenty normal healthy volunteers were selected and randomized into two groups of 10. The study was divided into three stages: baseline period (1 week), supplementation period (2 weeks), and follow-up period (1 week). During the baseline period the subjects consumed their normal diets and gave two fecal samples (in the beginning and at the end). During the supplementation period two fecal samples (after the first and second

supplementation week) were collected. During the follow-up period a fecal sample was collected at the end of the week. The fecal samples of 11 of the volunteers were positive for AFB_1, with levels ranging between 1.8 and 6 μg AFB_1/kg feces. For volunteers who were administered the probiotic preparation, there was a significant reduction in the level of AFB_1 after the second week of the trial, a reduction that continued during the follow-up period. There was no difference in the consumption of foods known to be sources of exposure to AFB_1 between the group receiving the treatment and the control group. These results suggest that the probiotic strains used in this trial have the ability to influence the fecal content of AFB_1. Clinical studies are currently underway in China in a population exposed to aflatoxins to further assess the possibility to prevent absorption of ingested aflatoxins by dietary probiotics.

VI. POTENTIAL HEALTH BENEFITS OF LACTIC ACID BACTERIA IN THE CONTEXT OF THIS APPROACH

During recent years a number of research reports have focused on lactic acid bacteria, in particular lactobacilli and bifidobacteria, which are used for the production of yogurt and various fermented milk products. It is generally accepted by consumers that such bacteria, when present in fermented dairy foods, have beneficial effects on health. Scientific evidence shows that specific strains of probiotic bacteria have well-documented health effects in humans.

Probiotic bacteria are microorganisms that have a beneficial effect on intestinal function and promote health. Over recent years dietary use of probiotic bacteria has gained popularity. Human intestinal microbiota is influenced by diet and microbiota, which in turn may influence our health. Intestinal bacteria contribute to mucosal integrity, metabolism, and immune status, both locally and systemically. For successful use, generally probiotic bacteria must survive in the environment in which they are intended to act and must reach the small intestine or colon alive. Such bacteria are considered safe,[20,21] and some even possess health benefits when they are nonviable.[22]

Probiotic bacterial strains used in mycotoxin detoxification can also have other beneficial health effects. For example, there is promising research suggesting that *Lb. rhamnosus* strain GG can decrease the risk of bowel cancer,[23] a major health problem in industrialized countries associated with diets high in animal protein, high in fat, and low in fiber. The most convincing evidence thus far has been received on *Lactobacillus casei* Shirota. Several mechanistic studies reported decreased urinary mutagen excretion and potential influence on absorption of toxins and harmful compounds after use of this strain. In clinical studies the prophylactic effects of oral administration of *Lb. casei* Shirota on the recurrence of superficial bladder cancer have been reported in Japan.[27] Recently, a large Japanese case-control study on the habitual intake of lactic acid bacteria and risk reduction of bladder cancer was conducted in the specific setting of home delivery of the product.[28] The results suggested that habitual intake of milk fermented with this strain reduces the risk of bladder cancer in the Japanese population. This result, combined with the mechanistic work and human studies, warrants further investigation in other countries and also to gain an understanding of the probiotic-toxin relationship in the gut. It is important that the bacteria used are of human origin, occurring naturally in normal human intestinal microbiota. Thus, modulation of the intestinal microbiota may offer one tool for reducing risks related to food contamination in the future.

Persistence in the intestine appears to be correlated with strong adhesion. Our screening studies suggest that a correlation exists between strong aflatoxin binding and strong adherence to the human intestinal tract, to intestinal epithelial cells, and to mucin secreted by the intestinal mucosa. Bacteria possessing good adherence properties in in vitro adhesion models, such as Caco-2 and human intestinal mucus glycoprotein, have been reported to lose this property when aflatoxin is bound.[24] This is important when considering applications in animals and humans, as contact with the intestinal mucosa is significantly shortened when either viable or nonviable bacterial cells have bound aflatoxins or other mycotoxins.

VII. FUNCTIONAL FOODS

In addition to their potential for in vitro detoxification or in vivo detoxification in animals, these bacteria possess great potential for the development of functional foods or effective dietary supplements for detoxification. By the incorporation of these probiotic bacteria into foods, health benefits can be achieved and functional foods developed.[20,21] The animal studies conducted suggest that lactic acid bacteria in a nonviable form can be applied to animal feeds to prevent the negative effects of mycotoxins in meat, egg, and dairy production by binding dietary mycotoxins and removing them from the body. A similar approach can potentially be used for human dietary treatment in areas with heavy mycotoxin contamination. Further studies should be conducted to apply this biotechnology to commercial animal feeds and the development of functional food products.

VIII. STUDIES ON TOXIN BINDING AND PROBIOTICS

Recent reports have increased the knowledge of bifidobacteria and their ability to bind mycotoxins. This may be an important area of research, especially in mycotoxin-contaminated areas where even human breast milk may contain significant amounts of aflatoxin or other toxins.[25] As bifidobacteria form 60–90% of the total healthy infant microbiota, members of this species may offer an important novel means of decontaminating the diet of breast-fed or formula-fed infants in areas of environmental mycotoxin contamination.

Recent work has also focused on the ability of lactic acid bacteria and bifidobacteria to bind heavy metals from the diet. In vitro studies show efficacy in such decontamination for specific strains of probiotic bacteria.[29] The removal of this new group of contaminants from foods and feeds needs further assessment and characterization. It also emphasizes the potential use of lactic acid bacteria and bifidobacteria in such applications and a need for rapid development of such biotechnology using already accepted food-grade microbial ingredients.

IX. CONCLUSION

The results reported suggest potential means of reducing the bioavailability of toxins from contaminated feeds, either by incorporation of specific bacteria into feeds or by inoculation of such bacteria into animals at birth. Development of this approach opens up prospects of reducing the bioavailability of food toxins and thus modifying their toxicity to animals. The practical application of the approach described requires further investigation, e.g., into the effects of food and feed matrices on the ability of the bacteria

to bind toxins and whether these bacteria can reduce the toxicity and carcinogenicity of the toxins to animals under farm conditions.

Successful application of this novel, low-technology approach will provide great benefits, especially in the developing countries where efforts to combat food contamination are generally limited by lack of resources and technology. It will impact the feed industry and animal farming, improving not only animal health and productivity in the agricultural industries, but also human health, by ensuring the safety of our food supply derived from animals.

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Prebiotics and Lactic Acid Bacteria

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I. INTRODUCTION

Biological functions of the human large intestine include waste storage and the absorption of water as well as essential minerals. However, because of a slow transit time, near-neutral pH and substrate availability, the colon harbors a very complex and diverse bacterial microflora.[1] The bacterial microbiota in the human large intestine is thought to compromise 95% of the total cells in the body, representing 10^{12} cells/g dry weight contents. Through the activities of the resident microflora, the colon plays a major role in host nutrition and welfare.[2] Dietary modulation of the human gut flora can be of great benefit to health. In recent years, the functional food concept has moved away from mineral and vitamin supplementation towards the situation where improved gut (microbial) functionality is the main current driving force. The colon is the most intensely populated region of the gastrointestinal tract and is therefore the main target for such dietary intervention.

The resident gut microbiota ferments a range of substances, mainly provided by the diet, that cannot be digested by the host in the small gut and are available for fermentation by the colonic microflora. These include resistant starch, nonstarch polysaccharides (dietary fiber), oligosaccharides, proteins, amino acids, etc. In a typical adult, around 80 g of food ingested each day reaches the large intestine and is therefore susceptible to fermentation by the gut flora. The two main types of fermentation that are carried out in the gut are saccharolytic and proteolytic. The main end products of carbohydrate metabolism are the short-chain fatty acids, acetate, propionate, and butyrate. These may be further metabolized systemically or locally to provide energy generation for the host. The end products of proteolytic fermentation include phenolic compounds, amines, and ammonia, all of which are toxic. The proximal colon (right side) is essentially a site of saccharolytic fermentation, whereas the more distal (left side) sees more proteolytic fermentation.

Dietary modulation of the human gut microbiota is a topical area of nutritional sciences. This is driven by the fact that the gastrointestinal tract, particularly the colon, is very heavily populated. Undoubtedly, certain gut species are pathogenic and may be involved in the onset of acute and chronic disorder. Bifidobacteria and lactobacilli are considered to be examples of health-promoting constituents of the microflora. Lactobacilli may aid digestion of lactose in lactose-intolerant individuals, reduce constipation and infantile diarrhea, help resist infections such as salmonellae, prevent traveller's diarrhea, and help in irritable bowel syndrome (IBS).[3] Bifidobacteria are thought to stimulate the immune system, produce B vitamins, inhibit pathogen growth, reduce blood ammonia and blood cholesterol levels, and help to restore the normal flora after antibiotic therapy.[4] Health-promoting effects of the microflora may include immunostimulation, improved digestion and absorption, vitamin synthesis, inhibition of the growth of potential pathogens, cholesterol reduction, and lowering of gas distension.[4] Harmful effects are carcinogen production, intestinal putrefaction, toxin production, diarrhea/constipation, liver damage, and intestinal infection. However, most bacteria in the gut are benign, with the possibility that some groups are beneficial. Bifidobacteria and lactobacilli are thought to belong to this latter category and are common targets for dietary intervention that improves health.

II. GENERAL ASPECTS OF PREBIOTICS

Dietary modulation of the human gut flora has been carried out for many years. In humans there are positive aspects to the gut fermentation which may improve certain aspects of host health. The microflora contains certain bacteria that can be perceived as health promoting as well as pathogenic. For instance, bifidobacteria and lactobacilli may help to improve resistance to gut infections by inhibiting the growth of harmful microorganisms (that may onset both acute and chronic gut disorder), reduce blood lipid levels, improve the immune response, and be involved in protection against gut cancers.[2,5] The definitive health outcomes and their mechanisms of effect are being gradually uncovered, and there is currently much interest in increasing numbers and activities of these bacteria in the large gut, preferably at the expense of more harmful species. The manner in which this can be achieved is through dietary supplementation.

An alternative approach has been investigated where the commensal bifidobacteria and/or lactobacilli are selectively promoted by the intake of certain nonviable substrates, known as prebiotics. Gibson and Roberfroid[4] first described a prebiotic as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." As diet is the main factor controlling the intestinal microflora, it is possible to modulate the microflora composition through foods. A prebiotic substrate is selectively utilized by beneficial components of the indigenous gut flora but does not promote growth of potential pathogens such as toxin producing clostridia, proteolytic bacteroides and toxigenic *Escherichia coli.* In this manner, a "healthier" microflora composition is obtained whereby the bifidobacteria and/or lactobacilli become predominant in the intestine and exert possible health-promoting effects (similar to the situation that prevails in the breast fed infant gut). For a dietary substrate to be classed as a prebiotic, three criteria are required: (a) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine, (b) it must be selective for beneficial commensal bacteria in the colon such as the bifidobacteria, and (c) the substrate should induce beneficial luminal/systemic effects within the host.

A range of substrates of dietary origin, or produced by the host, are available for fermentation by the colonic microflora. Through diet, resistant starch (RS) is the most quantitatively important.[6] Nonstarch polysaccharides (NSP) form the next largest contribution and include plant-derived substrates such as pectin, cellulose, hemicellulose, guar, and xylan. Sugars and oligosaccharides like lactose, lactulose, raffinose, stachyose, and fructo-oligosaccharides also escape absorption in the small intestine and are metabolized by species of colonic bacteria. Mucin glycoproteins produced by goblet cells in the colonic epithelium are predominant endogenous substances fermented in the colon. Related mucopolysaccharides such as chondroitin sulfate and heparin and pancreatic and bacterial secretions are also available for the intestinal microflora.[7] Finally, proteins and peptides originating in the diet, in pancreatic secretions, or produced by bacteria are also available in the colon,[8] although to a lesser extent than the carbohydrates.

The premise behind prebiotics is therefore to stimulate certain indigenous bacteria in the gut rather than introducing exogenous species, as is the case with probiotics. Ingesting a diet containing nondigestible carbohydrates that are selectively fermented by indigenous beneficial bacteria is the prebiotic principal. Any dietary component that reaches the colon intact is a potential prebiotic, but much of the interest in the development of prebiotics is aimed at nondigestible oligosaccharides such as fructooligosaccharides (FOS), *trans*-galactooligosaccharides (TOS), isomaltooligosaccharides (IMO), xylooligosaccharides (XOS), soyoligosaccharides (SOS), glucooligosaccharides (GOS), and lactosucrose.

On the contrary, it may be possible to intake prebiotics more naturally through the diet. Many fruit and vegetables contain prebiotic oligosaccharides such as FOS. Examples are onion, garlic, banana, asparagus, leek, Jerusalem artichoke. However, the likely situation is that levels are too low to have any significant effect. Our (unpublished) data indicate that at least 4 g/d but more preferably 8 g/d of FOS would be needed to significantly elevate bifidobacteria in the human gut. Hence, there exists much value in the approach of dietary fortification.

As the majority of bacteria resident in the gut microbiota are present in the colon, prebiotics are usually directed towards lower gut bacteriology. Any dietary material that enters the large intestine is a candidate prebiotic. This includes carbohydrates such as resistant starch and dietary fiber as well as proteins and lipids. However, current prebiotics seem to be confined to oligosaccharides that are non-digestible in the upper gut and seem to confer the degree of fermentation selectivity required, e.g., directed towards bifidobacteria.

Oligosaccharides are sugars consisting of between approximately 2 and 20 saccharide units, i.e., they are short-chain polysaccharides. Some occur naturally in several fruits and vegetables and are extractable, while others can be commercially produced through the hydrolysis of polysaccharides (e.g., dietary fibers, starch) or through enzymatic generation. The following oligomers have been suggested as having prebiotic potential:[9]

Lactulose
Fructooligosaccharides
Galactooligosaccharides
Soybean oligosaccharides
Lactosucrose
Isomaltooligosaccharides
Glucooligosaccharides
Xylooligosaccharides
Palatinose

A. Structure-Function Relationships

The list of recognized prebiotics represents a wide range of carbohydrate chemistry. Despite this, we still have very little understanding of the structure-function relationships in these molecules. Moreover, we do not have good comparative data on their fermentation properties (particularly at a microbial species level), and there is no predictive ability with respect to new carbohydrate structures isolated or synthesized. This is likely to hamper new product development based around the concept.

The prebiotic properties of carbohydrates are likely to be influenced by the following factors:

1. Monosaccharide composition: Recognized prebiotics are primarily built from glucose, galactose, xylose, and fructose. The prebiotic potential of oligosaccharides composed of other monosaccharides such as arabinose, rhamnose, glucosamine, and galacturonic acid is not known at the present time.
2. Glycosidic linkage: The linkage between the monosaccharide residues is a crucial factor in determining both selectivity of fermentation and digestibility in the small intestine. The current paradigm for the selective fermentation of prebiotics is the cell-associated β-fructofuranosidase isolated from bifidobacteria. If this model holds true for other oligosaccharides and bacteria, then the linkage specificity of the glycosidases will be very important. Maltose is not recognized as prebiotic and is metabolized by the human intestinal brush border glycosidases. Isomaltose and isomaltooligosaccharides, however, are prebiotic. Both are composed of α-glucosyl linkages, but the 1–6 linkage in the IMO renders them partially resistant to metabolism in the small intestine and confers selectivity of fermentation in the colon.
3. Molecular weight: Generally speaking, polysaccharides are not prebiotic.[10] Conversely, all known prebiotics have low molecular weight. Inulin has the highest molecular weight, but most of the carbohydrate in inulin has a degree of polymerization (DP) of <25, with an average around DP 14.[11] The effect of molecular weight on prebiotic properties can be seen from the fact that xylan is not selective, whereas xylooligosaccharides are.[12,13] Olano-Martin and coworkers have investigated this effect in more detail and found an increase in selectivity upon hydrolysis of dextran to IMO[14] and upon hydrolysis of pectins to pectic oligosaccharides.[15] The precise relationship between molecular weight and selectivity is not known at the present time for any polysaccharide/oligosaccharide system.

B. Increased Molecular Weight

Most current prebiotics are of relatively small DP, the exception being inulin. As discussed above, it is believed that the oligosaccharides must be hydrolyzed by cell-associated bacterial glycosidases prior to uptake of the resultant monosaccharides. It is, therefore, reasonable to assume that the longer the oligosaccharide, the slower the fermentation and hence the further the prebiotic effect will penetrate throughout the colon. For example, long-chain inulin may exert a prebiotic effect in more distal colonic regions than the lower molecular weight FOS, which may be more quickly fermented in the saccharolytic proximal bowel. This approach has led to industrial forms of inulin/FOS mixtures with controlled chain length distributions ("Synergie II," manufactured by Orafti, Tienen,

Belgium), which in theory should persist further in the hindgut. This approach might have great promise for making more persistent prebiotics.

C. Potential Food Applications

The current concept of a prebiotic is an oligosaccharide that is selectively fermented by bifidobacteria and lactobacilli.[4] Due to the difficulties of characterizing the colonic microflora at the species level, virtually all of the data on prebiotic properties of oligosaccharides are on microflora changes at the genus level. It would, however, be highly desirable to develop prebiotics targeted at particular species of *Bifidobacterium* and *Lactobacillus.* Such targeted prebiotics might be considered for several applications:

1. Synbiotics with defined health benefits: Many probiotic strains have been developed to have particular health benefits such as immune stimulation or anti-pathogen activity. In addition, commercial probiotic strains are selected for their survival characteristics such as resistance to acid and bile and their ability to be freeze-dried.[16,17] Availability of prebiotics targeted at these strains would enable the development of synbiotic versions with enhanced survivability and colonization in the gut.
2. Infant formulas: It has long been known that the gut flora of the breast-fed infant is dominated by bifidobacteria and that this is not the case for formula-fed infants.[18,19] This is thought to be one reason for the improved resistance to infection that the latter group experience. If prebiotics could be developed that have particular selectivity towards those bifidobacteria present in the guts of breast-fed infants, a new range of synbiotic formula foods could be envisaged.
3. Functional foods for the elderly: Above the age of about 55–60 years, fecal bifidobacterial counts have been shown to markedly decrease compared to counts of younger people.[20,21] This decrease in bifidobacteria is a cause for concern as the natural elderly gut flora may have become compromised through reduced bifidobacterial numbers, resulting in a diminished ability to resist colonization with invading pathogens. Prebiotics may be potentially utilized as a dietary intervention in the attempt to restore the youthful microflora balance of the gut in the elderly population, concurrently with indirectly providing antipathogenic protection.

As prebiotics exploit the use of nonviable dietary components to improve gut health, the range of foods into which they can be added is much wider than that for probiotics, where culture viability needs to be maintained. This has the advantage that heat stability or exposure to oxygen is not an issue. As such, virtually any carbohydrate-containing food is susceptible to supplementation. Examples are shown in Table 1.

III. HEALTH-RELATED ASPECTS AND APPLICATIONS

The prebiotic approach advocates the administration of nonviable entities. At present, most prebiotics are selected on the basis of their ability to promote the growth of lactic acid-producing microorganisms. Fructooligosaccharides, lactulose, and galactooligosaccharides are all popular prebiotics. In Europe the most success has been gained with fructooligosaccharides. In human studies, after a short feeding period, they stimulate bifidobacteria in the lower gut.[22] Similarly, lactulose is an efficient prebiotic, as

Table 1 Potential Applications for Prebiotics as Food Ingredients to Improve Gastrointestinal Health of the Consumer

Beverages and fermented milks
Health drinks
Bakery products
Table spreads
Sauces
Infant formulas and weaning foods
Cereals
Biscuits
Confectionery, cakes, desserts
Snack bars
Soups

demonstrated through the use of molecular probes in a human volunteer trial (Tuohy et al., 2001). In Europe, FOS, GOS, and lactulose have been shown to be prebiotics through numerous volunteer trials, as evidenced by their ability to change the gut flora composition after a short feeding period.[9] The Japanese market is more widespread. A recent volunteer trial was carried out at the University of Reading (Tuohy et al., 2001). Shortbread containing 7 g/d FOS was fed to human subjects and the effects upon fecal bacteria determined as compared to a placebo (FOS not added). The nature of the trial was a crossover approach in that volunteers took active and placebo shortbread, but neither they nor the investigators were aware of which was ingested. Moreover, the bacteriology was carried out using a (culture-independent) probing approach that relied upon differences in 16SrRNA profiles for the confirmation of identity. The data clearly show that the use of FOS exerted a profound effect upon bifidobacteria. A number of benefits can be ascribed to prebiotic intake (Gibson et al., 2002).

A. Protection Against Colon Cancer

Many common diseases of the human large bowel arise in the distal colon, particularly colon cancer.[23] Prebiotics have been postulated to be protective against the development of colon cancer.[24–28] The second most prevalent cancer in humans is colon cancer,[29] and it is thought that tumors arise 100 times more often in the large intestine compared to the small intestine.[30] For this reason, many researchers believe that the colonic microflora has an important role to play in the development of bowel cancer.[31] It is known that several species of bacteria commonly found in the colon produce carcinogens and tumor promoters from food components that reach the colon. Interest in a diet-mediated intervention in colon cancer arises due to the slow, progressive nature of the disease and the fact that we can influence colonic microbiology by diet. There have been several studies on the use of prebiotics in cancer prevention, mainly focusing on animal models.

It is thought that prebiotics may protect against the development of colon cancer through at least two mechanisms:

1. Production of protective metabolites: Butyrate is a common fermentation end product and is known to stimulate apoptosis in colonic cancer cell lines, and it is

also the preferred fuel for healthy colonocytes.[32,33] For these reasons it is generally believed desirable to increase the amount of butyrate formed in the large gut. Some prebiotics are known to have this effect,[14,34] although it must be borne in mind that lactobacilli and bifidobacteria do not produce butyrate. Known butyrate producers in the gut are clostridia and eubacteria.[6] Development of prebiotics that stimulate eubacteria but not clostridia would be a desirable enhancement.

2. Subversion of colonic metabolism away from protein and lipid metabolism. It is possible that prebiotics would induce a shift in bacterial metabolism in the colon towards more benign end products. An obvious target would be shift the metabolism of clostridia and bacteroides away from proteolysis to saccharolysis.

Lactic acid bacteria are believed to have inhibitory effects on several bacteria that produce carcinogenic enzymes and are themselves nonproducers. Moreover, prebiotics may indirectly modify the activities of enzymes produced by the lactic acid bacteria that are involved in carcinogenesis, such as azoreductases, nitroreductases, and β-glucuronidase.[35]

To date, few prebiotics have been evaluated in animal and human trials. Inulin has been shown to inhibit the formation of aberrant crypt foci in rats.[25] Human studies are low in number and tend to focus on fecal markers of carcinogenesis rather than being epidemiological in nature. FOS, GOS, and resistant starch have all been investigated in this regard. FOS has been found to reduce genotoxic enzymes concomitant with increasing bifidobacteria,[26] and resistant starch has been found to reduce sterols, secondary bile acids, and genotoxic enzymes, although no microbiological studies were performed.[28] A recent study on GOS, however,[36] found no significant changes in bifidobacteria or in markers of carcinegenesis. These results might at first sight seem anomalous, as GOS are known prebiotics.[37] However, the starting populations of bifidobacteria in the volunteers were rather high (9.2–9.4 log). It has been noted[38] that the magnitude of the response to prebiotics by bifidobacteria depends on the starting levels. It is apparent that we currently have an inadequate knowledge of the effects of various prebiotics upon risk of colon cancer, and more studies are needed to address this. Development of prebiotics with the goal of reducing biomarkers of cancer would, however, be desirable.

B. Effects on Pathogens

Good evidence for the success of prebiotics probably lies in their ability to improve resistance to pathogens by increasing bifidobacteria and lactobacilli. Lactic acid–excreting microorganisms are known for their inhibitory properties.[39] In humans, viruses, protozoa, fungi, and bacteria can all cause acute gastroenteritis. Metabolic end products such as acids excreted by these microorganisms may lower the gut pH to levels below those at which pathogens are able to effectively compete. Also, many lactobacilli and bifidobacterial species are both able to excrete natural antibiotics which can have a broad spectrum of activity. For the bifidobacteria, some species are able to exert antimicrobial effects on various gram-positive and gram-negative intestinal pathogens.[40] A recent study in mice showed that FOS and inulin protected against enteric and systemic pathogens and tumor inducers.[41] This includes the Verocytotoxin strain of *Escherichia coli* O157 : H7 and campylobacters. Viral infections play a major role, but bacteria are also of great significance.

Much effort is being expended on cleaning up the food chain from "farm to fork" or "plough to plate." However, organisms causing infection have their effects after the fork or

plate, i.e., in the gut. It may be that a rational way to reduce the food-poisoning burden is to fortify certain components of the intestinal flora such that it becomes much more resistant to invasion. This is achievable through the use of prebiotics that target bifidobacteria and/or lactobacilli. Taking this further, some other gut-related conditions more chronic than acute gastroenteritis, but also leveled at microbiological pathogens, may also be susceptible to prevention or treatment by altering the gut flora. Examples would include ulcerative colitis, bowel cancer, peptic ulcers, pseudomembranous colitis, and *Candida*-induced conditions.

C. Improved Calcium Absorption

There has been increasing interest in recent years in the possibility of increasing mineral (particularly calcium) absorption through the consumption of prebiotics. Although the small intestine is the principal site of calcium absorption in humans, it is thought that significant amounts are absorbed throughout the length of the gut, and consequently maximizing of colonic effects is desirable.

Several mechanisms have been postulated for increased calcium absorption induced by prebiotics,[42] although it is far from clear at the present time which (if any) actually operate in vivo. Fermentation of prebiotics such as inulin results in a significant production of short chain fatty acids (SCFA), leading to a reduction in lumenal colonic pH. This is likely to increase calcium solubility and overall levels in the gut. Phytate (myoinositol hexaphosphate) is a component of plants that reaches the colon largely intact.[43] It also forms stable, insoluble complexes with divalent cations, like calcium, rendering them unavailable for transport. Fermentation results in bacterial metabolism of phytate, thereby liberating calcium. It is postulated that a calcium exchange mechanism operates in the colon. In this system, SCFA enter the colon in a protonated form and then dissociate in the intracellular environment. The liberated proton is then secreted into the lumen in exchange for a calcium ion. Numerous animal studies have indicated that prebiotics increase absorption of calcium from the colon and decrease losses from bone tissue.[44] Very few human studies have been carried out, however. In one such study, feeding 40 g of inulin per day for 28 days to nine healthy subjects resulted in a significant increase in calcium absorption.[45] A more realistic 15 g of inulin, FOS or GOS, per day fed to 12 healthy subjects for 21 days resulted in no significant effect on absorption of calcium or iron.[46] In a more recent study, 12 adolescent boys (14–16 years) were fed 15 g f FOS per day for 9 days in a placebo-controlled trial against sucrose.[47] The data showed a 10.8% increase in calcium balance with no significant effect on urinary excretion.

D. Effects on Blood Lipids

There is intense interest in the food industry in developing functional foods to modulate blood lipids such as cholesterol and triglycerides. It is widely believed that elevated cholesterol levels in the blood represent a risk factor for coronary heart disease, with low-density lipoproteins (LDL) being of most concern.[48] There is also evidence that lactic acid bacteria may be able to reduce total and LDL cholesterol levels. The mechanisms by which lactic acid bacteria, and hence, indirectly, prebiotics, influence blood lipids are not clearly understood at the present time. It is possible that some lactic acid bacteria may be able to directly assimilate cholesterol. This has been hypothesized from some in vitro experiments but is a source of contention in that the data are conflicting and precipitation of cholesterol with bile salts at low pH may occur, giving misleading results. It has been

suggested[48,49] that propionate produced by bacterial fermentation of prebiotics inhibits the formation of serum LDL cholesterol. The difficulty with this hypothesis is that bacterial fermentation of prebiotics generally produces much more acetate than propionate, as the target lactic acid bacteria (lactobacilli, bifidobacteria) are not propionate producers. Moreover, acetate is a metabolic precursor of cholesterol and may therefore tend to increase, not decrease, serum levels. There is evidence that FOS decrease the de novo synthesis of triglycerides by the liver. The means by which this occurs is not fully understood, but the effect appears be exerted at the transcriptional level. It is also possible that prebiotics (such as inulin) can modulate insulin-induced inhibition of triglyceride synthesis.[49] Human studies on the lipid-lowering properties of prebiotics when consumed at a realistic (tolerable) dose are not clear-cut.[50] Results are inconsistent, and only FOS, inulin, and GOS have been studied in this regard. It is possible that other prebiotics might lead to a more consistent effect, although until the mechanisms behind the effect are clarified, this will remain speculation.

E. Immunological Effects

Lactic acid bacteria have long been considered to be immuno-modulatory and several commercial products on the marketplace have built upon this concept. LAB are thought to stimulate both nonspecific host defense mechanisms and certain cell types involved in the specific immune response. The result is often increased phagocytic activity and/or elevated immunological molecules, such as secretory IgA, which may affect pathogens such as salmonellae and rotavirus. Most attention in this respect has been diverted towards the intake of probiotics (lactic acid bacteria)[51,52] and interactions between cell wall components and immune cells. As prebiotics serve a similar endpoint to lactic acid bacteria (i.e., improved gut microbiota composition), similar effects may occur through their intake. A recent animal study showed that prebiotics had an impact on immune function.[53] The question arises whether increased immune function, even through nonpathogenic means, is a desirable trait. A more detailed understanding of the immunological responses to particular changes in the colonic microflora may aid in the development of prebiotics with more desirable benefits.

IV. CONCLUSION

The microflora of the gastrointestinal tract is key for the nutrition and health of the host. Microflora modulation can occur through diets that contain prebiotics. The approach of using diet to induce microbial change offers a very straightforward approach towards improved health that is consumer friendly and effective. However, if progress in the use of dietary intervention directed towards particular gut bacteria is to be exploited, a sound research base is required. Some areas of interest include:

- The application of advanced molecular procedures that help identify the gut microbial diversity as well as allow effective tracking of microflora changes in response to diet (it is likely that a large number of gut bacteria have not hitherto been characterized, and culture-independent methodologies may help overcome this).
- The prebiotic potential of dietary ingredients and identification of those foodstuffs that can be fortified and the optimal dose required.
- A definition of prebiotics that act at the species level and have a high degree of selectivity and contain multiple biological activities.

Whether certain target groups are more susceptible to the approach (elderly, weaning stage, formula-fed infants, hospitalized patients).

A determination of the health consequences associated with gut flora modulation.

In terms of new developments, it is important that the definitive health bonuses associated with prebiotic intake be determined. This is especially relevant given the broad applicability of their use. It is likely that prevention of acute gastroenteritis through fortification of certain gut microbiota components is an important aspect. Moreover, improved protection from more chronic gut disorders that have been associated with bacteria (inflammatory bowel disease, colon cancer, irritable bowel syndrome) may also be possible. It may also be the case that certain target populations, such as infants, the elderly, and hospitalized persons, are more susceptible to the approach. The health benefits that have been suggested are varied, but also very important. In addition to good human volunteer studies, we also need to enhance our mechanistic understanding of the health effects of prebiotics. Progress is being made in this area, and it is to be expected that the prebiotic approach to prevention of disease will be have a much stronger foundation. This will lead to better informed decisions by clinicians, nutritionists, and consumers.

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14

Lactic Acid Bacteria in Vegetable Fermentations

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I. INTRODUCTION

The fermentation of plant material is an ancient preservation method, the origins of which have been traced to Asia.[1] In Europe, a total of 21 different vegetables are fermented, in addition to an unspecified number of variably composed vegetable blends and fermented vegetable juices (2). The most common products in Europe and the United States are sauerkraut, cucumbers, and olives. Fermented olives are manufactured mainly in the Mediterranean region. The fermentation of these products is carried out in factories or on farms.[2,3] In Korea, kimchi is a traditional fermented vegetable food.[4]

According to Buckenhüskes and coworkers,[2] it is generally agreed that fermented plant products are the "food of the future." Factors supporting this idea include:

- The high degree of hygienic safety caused by repression of growth of pathogenic bacteria
- Products can be marketed as "natural" or "biological"
- Enrichment of desired metabolites such as L-lactic acid or amino acids
- Build-up of flavor compounds and destruction of negative flavor compounds such as glucosinolates
- Less energy input than most other methods of preservation
- Simple handling and storage without cooling
- Easy method for prehandling of raw material before further processing

Most fermented vegetable products including sauerkraut juice are still produced by spontaneous fermentation, which typically involves a succession of microbial populations.

The amounts of lactic acid bacteria (LAB) in fresh vegetables are very low (5–10). Lactic acid bacteria found on plant material are presented in Table 1.

The sequence of natural fermentation and storage of vegetables has been divided into four stages: initiation of fermentation, primary fermentation, secondary fermentation, and postfermentation.[12,13] Since the amount of LAB in the original bacterial population is at most 1%, the aerobic organisms and the facultatively anaerobic enterobacteria are active at the beginning of fermentation. The primary fermentation is dominated by LAB and yeast. Their growth rate depends on several factors, including the initial microbial population and the physical and chemical properties of the vegetables and the environment. Due to the acid production and low buffering capacity typical of most vegetables, the pH of the fermenting material drops quickly. Parallel to this, the redox-potential goes down because of respiration, microbial activity, and a change of atmosphere caused by CO_2 production. This results in advantageous selection for LAB.[12–14] Secondary fermentation and postfermentation are caused by spoilage bacteria, yeasts, or molds, which use residual sugars or fermentation acids as substrates.[12,13] In this chapter the lactic acid fermentation of sauerkraut and cucumbers is described in detail. The processing and microbiology of vegetable fermentations have been described elsewhere.[3,6,9,11,13–16]

II. CHARACTERISTICS OF LAB IN VEGETABLES

LAB that dominate vegetable fermentations belong to the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus.* Although species of *Enterococcus* and *Lactococcus* have been isolated at the initiation of fermentation, their significance in spontaneous fermentation has not been established.[13] The species normally found in fermented vegetables do not usually reduce nitrate, and the presence of fermentable carbohydrate is a prerequisite for their growth.[17–20] The LAB involved in the initiation of fermentation and in primary vegetable fermentations are shown in Table 2. Homofermentative strains of lactobacilli produce 85% lactic acid from glucose and heterofermentative strains lactic acid, CO_2, ethanol, and/or acetic acid in equimolar amounts.[21] Fructose can function as a hydrogen acceptor, and it is reduced to mannitol by heterofermentative LAB.[17] The phenotypic variability of *Lb. plantarum* has long been known. Genomic heterogeneity has also been shown by DNA/DNA homology studies.[17] Kleerebezem and coworkers[22] have described the complete genome sequence of *Lb. plantarum* WCFS1. *Lactobacillus pentosus* and *Lactobacillus paraplantarum* are new species related to *Lb. plantarum*, and they both are able to ferment xylose.[23] Strains of *Lb. sake* and *Lb. curvatus* are

Table 1 Lactic Acid Bacteria Associated with Plant Material

Lactobacillus brevis	*Pediococcus acidilactici*
Lactobacillus casei	*Pediococcus pentosaceus* (formerly *P. cerevisiae*)
Lactobacillus plantarum	
Lactobacillus arabinosus	*Enterococcus faecalis* (formerly *Streptococcus faecalis*)
Lactobacillus buchneri	*Enterococcus faecalis* var. *liquefaciens*
Lactobacillus fermentum	*Enterococcus faecium* (formerly *Streptococcus faecium*)
Leuconostoc mesenteroides	*Lactococcus lactis* (formerly *Streptococcus lactis*)

Source: Ref. [11]

Table 2 Lactic Acid–Producing Bacteria Involved in Vegetable Fermentations.

Genus and species	Fermentation type
Enterococcus faecalis	Homofermentative
Lactobacillus bavaricus	Homofermentative
Lactobacillus brevis	Heterofermentative
Lactobacillus fermentum	Homofermantative
Lactobacillus pentosus	Homofermentative
Lactobacillus plantarum	Homofermentative
Lactococcus lactis	Homofermentative
Leuconostoc mesenteroides	Heterofermentative
Pediococcus acidilactici	Homofermentative
Pediococcus pentosaceus	Homofermentative

Source: Adapted from Ref. [13]

phenotypically closely related,[17] and they are considered beneficial in sauerkraut. The racemase-deficient *Lb. bavaricus* strains are used as starter cultures in sauerkraut.[24]

Murphy and Condon[25] demonstrated that during aerobic incubation, *Lb. plantarum* produces H_2O_2, the accumulation of which represses its growth. LAB use manganese (Mn^{2+}) as a scavenger of toxic oxygen species, particularly the superoxide radical anion (O_2^-).[26] This is a unique defense mechanism of LAB associated with plants[11] and is reflected in the high internal manganese content of such bacteria.[26,27]

Leuconostocs are facultatively anaerobic LAB, which have complex growth factor and amino acid requirements. They produce CO_2, ethanol or acetic acid, and D-lactic acid from glucose.[18] Leuconostocs have been isolated from the plant surfaces, and they initiate fermentation in vegetable products more rapidly than other LAB or other competing bacteria. They produce slime in media containing sucrose.[28] *Ln. mesenteroides* is considered the dominant species in the early stage of sauerkraut fermentation, but other species, such as *Leuconostoc fallax*, may be present.[29]

Pediococci are facultatively anaerobic bacteria with complex growth factor and amino acid requirements. They ferment glucose to D,L-lactic acid. *Pediococcus pentosaceus* and *Pediococcus acidilactici* grow quickly in suitable media, and they are widespread on vegetable material. The acid-tolerant, low-temperature species *Pediococcus damnosus* and *Pediococcus parvulus* grow slowly and require the most anaerobic conditions for growth.[19]

A. Antimicrobial Fermentation End Products

Preservation of vegetables by lactic acid fermentation is based on the production of organic acids, leading to a rapid drop of pH (see also Chapter XX). The organic aicds and low pH will, in combination with NaCl, inhibit the growth of undesirable gram-negative organisms.[16] LAB are also known for their ability to produce bacteriocins[30,31] although their in situ production remains to be proven. Microorganisms display varied tolerances to acids. The inhibitory effect of acids have been compared on the basis of pH, concentration, chain length, type, and degree of branching to inhibit or kill a wide

variety of microorganisms. The effective use of an acidulant depends upon the dissociation constant, pKa, which in most organic acids lies between pH 3 and 5.[32] The antimicrobial effect of fermentation acids is based on the concentration of the undissociated form of the acids in synergy with a low pH.[33–35] Upon entering the cell, the undissociated acid dissociates into its anion and proton because of the somewhat neutral intracellular pH. This reduces the intracellular pH to a level that will rapidly kill the cell unless the ions are excreted again by active transport, a process which requires energy.[36] Anions have antimicrobial activity as well. The acid tolerance of *Lb. plantarum* is likely the result of its ability to maintain pH homeostasis even at low external pH.[37] Oxidative yeasts are able to utilize organic acids as a carbon and energy source and can cause spoilage through deacidification of fermented plant material.[11] Savard and coworkers[38] demonstrated that the growth of spoilage yeasts isolated from fermented vegetables was not prevented by acidification lactic acid alone to pH 3.74 and that the growth was inhibited by propionic acid alone or together with acetic acid.

The accumulation of CO_2 in fermented plant products is the result of an endogenous respiration of the plant cells combined with microbial activities.[39] The overall effect of CO_2 on microorganisms is an extension of the lag phase of growth and a decrease in the growth rate during the logarithmic phase. The inhibitory effects of CO_2 on microorganisms in a culture medium or food system are dependent on many factors, such as partial pressure of, CO_2, the concentration of, CO_2, storage temperature, and the type of microorganism. Although the bacteriostatic effect of CO_2 has been known for many years, the precise mechanism of its action is still not clearly understood.[40] Gram-negative bacteria are generally more sensitive to CO_2 than gram-positive bacteria,[41] and lactobacilli are generally among the most resistant bacteria.[42]

III. FERMENTATION PROCESSES

A. Sauerkraut

The developments in plant breeding have produced cultivars of cabbage (*Brassica oleracea*) with variable suitability for lactic acid fermentation.[43] The concentration of fermentable sugars, glucose, fructose, and sucrose, is between 3 and 9% in fresh matter.[16,44,45] Before processing, the cabbage heads are trimmed, the cores are removed, and the cabbage is shredded. Salt is mixed with the shredded cabbage, and the brine begins to form immediately. The containers are sealed hermetically and pressed with weight. Fermentation is allowed to proceed for as little as a few weeks to as long as a year before packaging.[16] The lactic acid fermentation is initiated by *Ln. mesenteroides* and followed by *Lb. brevis, P. pentosaceus*, and finally by *Lb. plantarum*.[46]

Buckenhüskes and coworkers[7] confirmed that the fermentation was started by *Ln. mesenteroides*, and after 7 days the LAB flora had changed and was dominated by *Lb. plantarum*, *Lb. brevis*, *Lb. sake* and *Lb. curvatus* strains. *Ln. mesenteroides* produces lactic and acetic acids and CO_2, which rapidly lower the pH, thus limiting the activity of undesirable microorganisms and enzymes that might soften the shredded cabbage. The CO_2 replaces air and creates an anaerobic atmosphere, which is important to prevent the oxidation of ascorbic acid and to avoid darkening the natural color of the cut cabbage.[15]

The dominant LAB flora alters according to the temperature and salt content. The ideal temperature and NaCl concentration for sauerkraut fermentation are 18°C with and 1.8–2.25% NaCl.[16] Low temperature, even 5°C, allows low acid production mainly

by *Ln. mesenteroides*, which grows at a lower temperature than the other LAB. A rise in temperature alters the fermentation to homofermentation.[7,46] At 32°C *Lb. plantarum* and *P. pentosaceus* become dominant and the rate of acid formation is increased.[46] Stamer and coworkers[47] and Yildiz and Westhoff[48] demonstrated that the growth of *Ln. mesenteroides* was more repressed by 2.25% NaCl concentration in cabbage juice than the growth of *Lb. brevis*, *P. pentosaceus*, and *Lb. plantarum* strains. In commercial raw sauerkraut the pH varies between 3.2 and 3.4, the titrable acidity from 2.1 to 3.3%, and the salt content from 1.3 to 2.3%.[44]

Antimicrobial compounds in fresh and heat-treated cabbage juices have been studied recently by Kyung and coworkers.[49–51] The inhibitory compounds were identified as methyl-methanethiosulfinate and methyl-methanethiosulfonate, which are hydrolyzed from a nonprotein sulfur amino acid, *S*-methyl-L-cysteine sulfoxide, enzymatically or thermally, respectively.

Chopping, cooking, freezing, and fermentation of cabbage bring glucosinolates, sulfur-containing glucosides in cabbage, into contact with the enzyme myrosinase, which results in their breakdown.[52] During 2 weeks of sauerkraut fermentation, glucosinolates are decomposed[53,54] and different types of breakdown products are formed. Isothiocyanates and indole-3-carbinoles are considered to be protective, anticarcinogenic compounds,[55–57] and isothiocyanates together with allyl cyanide have been the predominant breakdown products of glucosinolates in sauerkraut.[53]

Experimental results of Oh[58] demonstrated that daily consumption of 300 g of raw sauerkraut or raw kimchi inhibited the synthesis of enzyme and/or enzyme activity, which mediates the conversion of procarcinogens to proximal carcinogens involved in colon cancer.

B. Cucumber

Cucumber fermentation has been reviewed by Etchells and coworkers,[6] Sandhu and Shukla,[59] and Harris.[16] Cucumbers are fermented in brine with an initial salt concentration that can range from 5 to 8% at ambient temperature. The fermentation is commonly completed within 2–3 weeks when the lactic acid concentration is approximately 1.1% and the final pH is about 3.3–3.5.[16] The initial brine strength may vary, depending on the individual pickling company.[6] In spontaneous fermentation of low-salt cucumber brines, great variations in the composition of dominant flora and fermentation acids were observed.[60] At the beginning of spontaneous fermentation, there is a rapid growth of gram-positive and gram-negative bacteria and yeasts. Initial load, growth rates, salt and acid tolerances are factors that are responsible for the sequence appearance of the LAB. During the fermentation, *Ln. mesenteroides*, *P. pentosaceus*, and *Lb. plantarum* are generally present.[11] *Lb. plantarum* is usually the dominant species at the end of the cucumber fermentation regardless of the original inocula.[61] Fermentation is affected by the chemical composition of cucumbers, which varies with size. Large cucumbers have been suggested to be more prone to secondary fermentation and bloater formation due to their higher sugar concentration.[62] Nutrients diffuse from solid vegetables into the brine, where they are accessible to microorganisms. The brine also serves to distribute the microorganisms present throughout the fermentation vessel.[11] LAB can enter and grow within cucumbers after they are brined,[63] whereas yeasts are unable to enter through stomata, presumably because of their larger size.[64]

IV. DEFECTS IN FERMENTED PRODUCTS

Slimy or ropy kraut has been recognized as a defect for many years. It is generally the result of dextran formation caused by *Ln. mesenteroides* and is a transitory problem since the dextrans are utilized by the other LAB. Slimy kraut caused by pectinolytic activity is permanent in effect.[15] However, the production of slime in sauerkraut has never been thoroughly investigated.[16]

Bloater formation of fermented cucumbers can be prevented by purging CO_2 from brines.[13] Bloater damage in brined cucumbers has been attributed to gas production by yeasts.[65] One of the sources of CO_2 is the decarboxylation of malic acid by *Lb. plantarum*,[66] a principal organic acid of cucumbers.[67] McFeeters and coworkers[68] showed that the amount of CO_2 produced is sufficient to cause bloater formation. The use of malolactic-deficient *Lb. plantarum* starter has prevented bloating in brined cucumbers.[68] Heterofermentative LAB have not been recommended as starters for cucumber fermentation because of CO_2 production.[11,65] The cucumber blossoms may carry high numbers of molds, which have caused quality problems, such as softening of cucumbers by pectolytic enzymes.[6]

V. STARTER CULTURES FOR FERMENTED VEGETABLES

Starter cultures applied in vegetable fermentation must possess appropriate and specific characteristics depending on the properties of the fermented commodity and the characteristics desired in the final product.[1] Requirements for LAB to be used for vegetable and vegetable juice fermentation have been presented by Buckenhüskes[1] and Fleming et al.[69].

Buckenhüskes and coworkers[7] demonstrated that inoculation of sauerkraut with *Ln. mesenteroides* resulted in a change of the dominating flora after 7 days as the pH went below 4.1. In sauerkraut inoculated with *Lb. plantarum* the total counts of LAB were higher than in the *Ln. mesenteroides* sauerkraut. The flavor and aroma substances were absent from *Lb. plantarum* sauerkraut. The residual sugar concentration in *Lb. plantarum* sauerkraut was about 1.8%, whereas in spontaneously fermented sauerkraut the sugars were exhausted. Fleming and McFeeters[44] also found that samples collected from commercial sauerkraut were completely fermented and no fermentable sugars could be detected. Delclós[70] used a mixed starter culture composed of *Ln. mesenteroides* and *Lb. pentosus* in sauerkraut fermentation. The product resembled spontaneously fermented sauerkraut, and the starter culture repressed the growth of *Listeria monocytogenes.*

Historically, *Lactococcus lactis* subsp. *lactis* has not been associated with vegetable fermentations, however, it has been isolated from sauerkraut.[71] *Lc. lactis* species isolated from vegetables have been found to produce nisin[71] or a bacteriocin similar to nisin,[72,73] which was shown to have a wide spectrum of activity not only towards a variety of LAB, but also to *Staphylococus aureus* and *L. monocytogenes.* Breidt and coworkers[74] showed that nisin is able to delay the growth of homofermentative LAB in brined cabbage fermentation. Harris and coworkers[75] used nisin-resistant *Ln. mesenteroides* and a nisin-producing *Lc. lactis* subsp. *lactis* as a paired starter culture in sauerkraut fermentation. Nisin was detected in sauerkraut within 24 hours, and the levels produced were sufficient to retard the onset of the growth of nisin-sensitive *Lb. plantarum* ATCC 14917.

Etchell and coworkers[76] compared the growth and acid production of pure cultures of *P. pentosaceus*, *Lb. plantarum*, and *Lb. brevis* and their mixture in sterilized and

naturally brined cucumbers. *P. pentosaceus* grew promptly and delayed the onset of the growth of *Lb. plantarum* when applied as a mixed starter. *Lb. brevis*, however, attained a relatively low population level and was later shown to cause bloating of cucumbers.[65] Fleming and coworkers[77] demonstrated that the inhibition of the growth of *Lb. plantarum* in mixed culture was caused by substances produced by the tested *P. pentosaceus*. It was concluded that the inhibitory interaction of strains in mixed starter cultures should be taken into consideration when strains are selected. Bacteriocin producing *Lb. plantarum* LPCO10 starter predominated indigenous microflora in green olive fermentation at application level of 10^5 cfu/mL whereas a non–bacteriocin-producing derivative *Lb. plantarum* 55-1 could not be isolated after 7 weeks.[78] This suggests that the production of antimicrobial substances plays an important role in other fermentations of plant material. The use of starter cultures in vegetable juices has become popular, except for sauerkraut juice, which is traditionally pressed from sauerkraut. The main target is to produce acid with little effect on the aroma.[14]

Gardner and coworkers [79] evaluated development of various LAB during fermentation and storage phases in vegetable mixtures of carrot, beet, and cabbage. The mixed cultures had more uniform fermentation patterns than pure cultures. The selected starter, consisting of *Lb. plantarum* NK 312, *P. acidilactici* AFERM 772, and *Ln. mesenteroides* BLAC, produced good sensory quality and repressed the growth of yeasts. The selected starter was tested by Savard and coworkers,[80] who demonstrated that by increasing the ratio of *Ln. mesenteroides*, the proportion of acetic acid increased and lactic acid decreased. In addition, the counts of LAB were lower after 30 days of storage.

Defined starter cultures have been developed to exploit metabolic activities not necessarily present in the "spontaneous" microbiota such as formation of exclusively L-lactic acid or the reduction of nitrate and nitrite.[81] The ratio of at least 75% of L-lactic acid in vegetable juice has been increased by a culture composed of strains of *Lactobacillus salivarius*, *Lactobacillus casei*, *Lactococcus lactis*, and *Lactococcus cremoris* or a mixture thereof.[82] Another single strain starter *Lb. bavaricus* ATCC 31063 has produced sauerkraut and pickled cucumbers in which the lactic acid was 90% L-isomer.[83]

Vegetables naturally accumulate nitrate, and some types of vegetable contain larger amounts than others. Nitrate may act as a source of toxic *N*-nitroso compounds.[84] The ability of a vegetable starter to reduce nitrate and nitrite may be beneficial for the quality of the end product.[14] However, Gierschner and Hammes[85] showed that *Lb. plantarum* and *Lb. pentosus* were unable to reduce nitrate in vegetable juice. Andersson[84] demonstrated that the gram-negative flora in carrots was responsible for nitrate reduction at the beginning of fermentation with or without *Lb. plantarum* inoculant and that the LAB present were unable to affect the nitrate content. The *Lb. plantarum* strains 92H and 90H and *Lactobacillus delbrueckii* strain 37H tested by Hybenová and coworkers[86] efficiently reduced nitrate in sterile cabbage and carrot juice.

Some toxicological characteristics and outbreaks of food poisoning are associated with the biogenic amines histamine and tyramine. Silla Santos[87] has reviewed the physiological effect of biogenic amines present in foods. Among others, genera of Enterobacteriaceae and Bacillaceae as well as species of *Lactobacillus*, *Pediococcus*, and *Streptococcus* are reported to be capable of decarboxylating one or more amino acids.[1] Kalač and coworkers[45] found that in spontaneously fermented sauerkraut, prepared with 2% (w/w) salt and fermented at 15°C for 14 days, the biogenic amine concentration increased significantly during 12 months of storage at 5–6°C. Tyramine was present at the highest levels, followed by putrescine and cadaverine.

The formation of biogenic amines has been repressed by the use of *Lb. plantarum* inoculant compared with spontaneous fermentation.[88–90] The recommended dosage was at least 5×10^6 cfu/g.[89,90] Kalač and coworkers[88] showed that inoculation of sauerkraut with pure cultures of *L. casei*, *P. pentosaceus*, or *E. faecium* at 22°C for 14 days did not inhibit the formation of biogenic amines during storage of six months at 5–6°C compared with spontaneous fermentation, but when they were added as mixed culture together with *Lb. plantarum* (Microsil, Medipharm), the biogenic amine levels were significantly lower.

Andersson[91] found that in fermentation of carrot, beet root, and a vegetable mixture of Swedish turnip, cabbage, and bell pepper with *Lb. plantarum* (10^6 cfu/g), the biogenic amine concentrations were considerably lower than those known to cause food poisoning, between 1 and 15 mg/kg of product.

In in vitro tests *Lactobacillus buchneri* and *Enterococcus faecium* showed tyrosine decarboxylase activity.[89] *Lb. buchneri* has been isolated from high histamine cheeses, whereas *Lb. brevis* isolated from cheese was found to produced tyramine.[21]

VI. CONCLUSIONS

LAB in vegetable fermentations are beneficial for the sensory and hygienic quality of the final products. The desired composition of the fermentation products can be influenced by variations in the salt concentration, temperature, fermentation time, and by the use of starter cultures. The development of starter cultures can aid in the economic improvement of fermentation processes as well as the safety and health aspects of the products.

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15

Lactic Acid Bacteria in Cereal-Based Products

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I. SUMMARY

Cereal-based foods are a major source of inexpensive dietary energy and nutrients worldwide. Cereal grains readily support the growth of microorganisms, including lactic acid bacteria (LAB), provided water and hydrolytic enzymes are present. Certainly many cereal foods, such as boiled or steamed rice, porridge, pasta, cookies, etc., are made without any fermentation process, and when fermentation is used alcoholic fermentation by yeast prevails, as in breadmaking and brewing. However, alcoholic fermentation of cereals often also involves lactic acid fermentation, and a mixed flora occurs. A number of cereal-based foods are characteristically fermented by lactic acid bacteria, such as the European sour rye bread, various Asian flat breads, and numerous types of fermented sour porridges, dumplings, and non- or low-alcoholic beers common in Africa, South America, and elsewhere. In these applications alcoholic fermentation may also have a role, but the lactic acid bacteria contribute to the technological and nutritional benefits, as well as affecting the flavor and keeping properties of the products.

Most fermented cereal-based foods are heat treated after fermentation and the bacteria are killed. However, there are also cereal-based foods that contain live lactic acid bacteria, both traditional lactic-fermented foods and novel applications. This chapter discusses some aspects of the traditional and novel role of lactic acid bacteria in the production of cereal-based foods.

II. CEREALS AS A SUBSTRATE

Cereals are, in general, a good medium for microbial fermentations. They are rich in polysaccharides, which can be used as a source of carbon and energy by microbes in

fermentation (Table 1). The major polysaccharide in cereals is starch, which becomes available to microbes after hydrolysis. The level of free sugars in fully matured sound grains may be only 1–3%, but even this supports the initiation of the fermentation process, and endogenous amylases will produce more from the polysaccharides in favorable fermentation conditions. In rye the contents of free sugars and amylolytic enzymes are higher than in other cereals, and this may partly explain the association of rye and sourdough baking. Endogenous cereal enzymes, added malt, or enzymes can be used to break down the starch to simple fermentable sugars, i.e., maltose and glucose. Lactic acid bacteria capable of utilizing starch are also known and may be present in cereal fermentations.

Besides carbohydrates, cereals also contain minerals, vitamins, sterols, and other growth factors, which support growth of microbes, including fastidious lactic acid bacteria.

Cereal grains normally carry an indigenous microbial flora composed of a variety of different microbes, such as molds, enterobacteria, aerobic sporeformers, etc., all of which compete for nutrients. Since no pasteurization can be generally applied without affecting the technological properties of starch and protein, a vigorous starter flora of lactic acid bacteria is required for successful fermentation.

III. LACTIC ACID FERMENTATION IN WET-MILLING

Dry cereal grains, being dry and hard nutritive packages, can be eaten only after milling or grinding and mixing with water. Hydrolysis by endogenous enzymes and fermentation by various microbes, including lactic acid bacteria, is often an unavoidable and integral part of the aqueous stage of cereal food preparation. For example, soaking of grains in water prior to wet-milling gives rise to various fermentation processes, including the lactic acid fermentation that often prevails in the end. Table 2 lists some functions of lactic acid fermentation in cereal food processes.

Soaking of grains in water prior to wet-milling is customary when corn, sorghum, or millet is ground in traditional food processing. Soaking softens the grain endosperm and greatly reduces the work input required for grinding. Penetration of water into the interior

Table 1 Compositional Data for Whole Dehulled Cereal Grains

Constituent	Content (%, dry matter basis)
Polysaccharides (total)	70–80
Starch	45–77
Dietary fiber (as nonstarch polysaccharides + lignin)	9–15
Low molecular weight carbohydrates (total)	2–5
Fructose	0.1–0.4
Glucose	0.1–0.5
Sucrose	0.5–2
Raffinose	0.2–0.7
Protein	8–15
Lipids	2–6
Ash (minerals)	1.5–3

Source: Ref.[1]

Table 2 Some Functions of Lactic Acid Fermentation in Cereal Food Processing

Operation	Material mixed with water	Principal purpose	Side effect or simultaneous reaction	Examples of a typical products	Ref.[a]
Soaking of grains prior to wet-milling	Whole grains	Softening of grain endosperm	Lactic acid fermentation, control of undesired micro-organisms	Ogi, agidi, koko, mawè	[2,5,6]
Slurrying or dough making after wet-milling	Wet starchy material from wet-milling	Separation of hulls etc. from the starchy endosperm	Flavor production, control of undesired organisms	Ogi, agidi, kenkey, mawè	[2,5,6]
Slurrying after dry milling	Coarse meal from dry-milling	Separation of hulls etc. from the starchy endosperm	Lactic acid fermentation, control of undesirable organisms	Kiesa, flummery	[9,11]
Doughmaking for bread	Flour	Aeration of dough	Acidification, flavor production, increase of mold-free time, control of α-amylase activity	Sourdough (rye) bread	[12]
Malting	Malting barley	Germination, release of nutrients, increase of α-amylase activity	Control of undesirable organisms	Barley malt	[8]
Brewing	Malted or unmalted cereal	Ethanol and flavor production	Acidification, flavor production	Country beers, boza, lambic beer	[13,14,16]
Brewing	Boiled corn/sorghum meal	Lactic acid, flavor production	Control of undesired organisms	Mageu	[14,15]
Cooking a gruel	Maize or sorghum	Lactic acid, flavor production	Control of undesired organisms	Togwa	[17,18]
Cooking a gruel	Oat bran	Proliferation of probiotic strains	Acidification, flavor production	Vellie	[1]

[a]Data from: Ref.[1,2,5,6,8,9,11–18]

of the kernels takes hours, and simultaneous fermentation occurs as an inevitable side effect, especially as the temperature cannot be controlled in normal applications. The fermenting microorganisms originate from the surface of the kernels and from other sources such as the steeping vessel and other equipment.[2–4] The resulting wet starchy material continues to undergo fermentation and carries the sour flavor, which is typical of the indigenous foods cooked from the fermented slurries.[5,6]

Lactic acid fermentation can also occur when the flour from dry-milling or the starchy material from wet-grinding is slurried without temperature control. This is common in the preparation of many tropical staple foods. Initially a short period of growth by fungi and bacteria belonging to Enterobacteriaceae may occur, followed by a lactic fermentation and alcoholic fermentation.[2] In such processes lactic acid bacteria inhibit pathogenic and spoilage organisms by several mechanisms, such as the production of organic acids, hydrogen peroxide, and antimicrobial substances, as well as by lowering pH and oxidation-reduction potential.[7] Lactic acid bacteria starter cultures have also been studied for controlling undesirable microbial growth in malting.[8]

When oats were prepared for food, the separation of hulls was formerly achieved by slurrying stone-ground oats with water. The procedure enabled the hulls to be strained from the surface, whereas endosperm particles sedimented. The slurry underwent simultaneous sourdough-type fermentation, and this was favored by adding rye sourdough.[9] The sour starchy sediment was used to cook indigenous fermented porridges and gruels, such as the Welsh flummery and Karelian kiesa.[10,11]

IV. LACTIC ACID BACTERIA IN BREADMAKING

A. Functions of Sourdough

Aeration of dough and bread probably was the principal reason for using sourdoughs from a historical perspective and the procedure predominated until specially prepared baker's yeast became available in the nineteenth century.[19] Easy access to effective and low-cost baker's yeast favored the use of wheat in baking and enabled extensive leavening of wheat doughs, a property only provided by wheat gluten. However, the use of sourdough was not eliminated in the baking of rye bread and various flat breads, although the aerating function of the sourdough is not necessarily its main function any longer.

The benefits of lactic acid fermentation are pronounced in nonwheat baking, particularly in rye bread making. Whereas sourness in white wheat bread induces a flavor not accepted to all people, sour rye bread is favored over nonacidified rye bread in northern, central, and eastern Europe. Dough for the various flat breads made in parts of Asia and Africa is often fermented in a process resembling that used for sour rye bread in Europe. Sourdough is also used for nonwheat breads or cakes, such as injera and kisra flat breads made from sorghum and other local cereals in Ethiopia and Sudan. Indian idli, made from rice and black gram, and puto, another steamed rice cake used as a snack in the Philippines, are also examples of breads or cakes made with sourdough.[20]

The functions of sourdoughs in breadmaking are listed in Table 3. The aeration occurring in a sourdough is caused primarily by the yeast. Heterofermentative lactic acid bacteria may also have a role.[21,22] In an Indian rice/legume-based aerated cake-type food, idli, heterofermentative lactic acid bacteria (*Leuconostoc*) were reported to be the micro-organisms responsible for leavening.[23] There is also some consumer interest in traditional breadmaking without added baker's yeast.[22,24]

Table 3 Functions of Sourdough in Breadmaking

Leavening action by yeast growing in association with lactic acid bacteria:
Dough easier to bake
Bread crumb softer and more palatable
Modification of flour components such as swelling and partial hydrolysis of protein and Polysaccharides:
Improvement of baking properties of rye dough
Improvement of crumb properties of rye bread
Control of excessive enzymatic acitivity of rye flour, especially α-amylase
Delay of starch degradation in wheat breads by using amylolytic strains
Control and inhibition of contaminating or spoiling flora during fermentation and in the end product due to organic acids and low pH, and possibly other mechanisms:
Elongation of mold-free time of bread
Prevention of growth of *Bacillus subtilis*, the rope-causing organism
Accumulation of flavor components such as lactic and acetic acids and other fermentation products
Increase of mineral bioavailability through degradation of phytate
Characterization of the product by a natural image; greater versatility, local and regional products
Suggested modification of starch structure leading to lower glycemic index values of wheat bread

In addition to the technological benefits and flavor, sour bread is characterized by better resistance to microbiological spoilage by molds and rope-forming bacilli.[25,26] The technological benefits of sourdough procedures in traditional rye breadmaking include the suppression of high endogenous activity of α-amylase and other enzymes and an increase in swelling of pentosans, which in turn improve gas retention.[27–29]

Modification and partial hydrolysis of proteins in wheat sourdoughs also occurs, primarily due to endogenous enzymes present in the flour.[30] Acidification by lactic fermentation may also contribute to the technological properties of doughs made from other nonwheat cereals, although such functions do not appear to have been the subject of published studies.

Sourdough technology provides possibilities for elongation of shelf-life time without added antimold or antirope or staling-inhibiting agents. These functions of sourdough have become even more important because of the negative consumer response to additives. The major antimicrobial compound in sourdough is acetic acid. Lactic acid reduces pH and by doing so increases the percentage of undissociated acetic acid, which is much more fungistatic than acetate. This concept is supported by experimental work (e.g.,[26,31,32]). However, other compounds may have a role too. Corsetti et al.[25] suggested that caproic acid formed by sourdough lactobacilli contributed to antifungal function. Lavermicocca et al.[33] found new antifungal compounds produced by a *Lactobacillus plantarum* strain. This Italian group also identified a bacteriocin-like substance from a *Lactobacillus* strain and found it to be active against a *Bacillus subtilis* strain.[34] Gänzle et al.[35] reported a heat-labile antibiotically acting compound, reutericyclin, to be formed by a *Lactobacillus reuteri* strain isolated from sourdough. A review of the inhibitory substances produced by lactobacilli isolated from sourdoughs was recently presented by Messens and De Vuyst.[36]

The potential of using amylolytic strains of lactobacilli in sourdoughs in order to inhibit staling by decreasing retrogradation through the enzymatic modification of starch has also been suggested.[37] In terms of nutrient bioavailability, it is obvious that phytate

degradation and release of minerals occurs in sourdough baking due to increased phytate solubility at low pH and endogenous phytase activity in flour.[29,38] It has also been reported that starch bioavailability is reduced by the use of sourdough and the glycemic response to starch is retarded.[39] A recent study confirmed that bread made with sourdough and containing lactic acid produced during fermentation has the ability to lower the postprandial glucose and insulin responses in humans.[40] The authors suggested that the underlying mechanism was an interaction between starch and gluten formed in dough.

B. The Sourdough Fermentation Process

A good sourdough is of great value technologically, and therefore rebuilding systems based on a previous batch developed. Repeated rebuilding of sourdough, or back-slopping, gives rise to selective enrichment of microorganisms adapted to the ecosystem. In sourdoughs the lactobacilli predominate, although leuconostocs and pediococci have also occasionally been reported to be present. Yeasts are present unless the fermentation is carried out at an elevated temperature that inhibits yeast growth. The contaminating flora such as molds, enterobacteria, etc. may be initially high but will be suppressed by lactic acid bacteria during the sourdough fermentation.

During sourdough fermentation the lactic bacteria and the yeasts multiply and metabolites accumulate. Figure 1a shows development of temperature, pH, titratable acidity, and colony counts of lactic acid bacteria and yeast during a batch-type industrial wholegrain ryemeal sourdough breadmaking process inoculated with 3% sourdough from a previous batch. Figure 1b shows the accumulation of lactic and acetic acids in the same process. In a fully developed wholemeal rye sourdough, the lactic acid content may exceed 1% and that of acetic acid may be 0.05–0.2%.

Whole cereal grains and 100% extraction rye flour may contain up to 10^2–10^5 CFU/g of unspecified bacteria and up to 10^2–10^3 CFU/g of lactic acid bacteria. Inoculation of the sourdough with 1–5% of a starter from the previous batch increases the number of lactic bacteria to 10^7 CFU/g or higher (Fig. 1a). This gives few possibilities for the growth of contaminating organisms, including those present in the flour. An obvious source of in-house inoculum is also the dough-handling equipment, which is difficult to clean, especially in the case of slimy and sticky rye sourdough. In a fully fermented sour rye sponge there may be more than 10^9 CFU of lactobacilli per gram. The number of yeast cells may be 50–100 times lower. Most sourdough processes are batch processes, although continuous propagation systems for large-scale industrial rye bread production have also been developed and are operating in Europe.

Traditionally the scientific literature on lactic acid bacteria in cereal-based foods has been derived from studies on sour rye bread, which is typical of central, northern and eastern Europe. Much of the earlier work was reviewed by Spicher and Stephan[41] in their book on sourdough technology. Lönner and Ahrné[42] reviewed applications of lactobacilli in baking. Later reviews were made by Gobbetti,[43] Hammes and Gänzle,[29] and Vogel et al.[44] Information on the microbiology of sourdough in various baking procedures is also included in the reviews by Caplice and Fitzgerald,[45] FAO,[20] Holzapfel,[4] and Blandino et al.[46]

C. Microecology of Sourdoughs

A typical stable sourdough is a microecological system that contains one to three major species of lactobacilli and a yeast, normally *Candida milleri*. Studies on the microecology

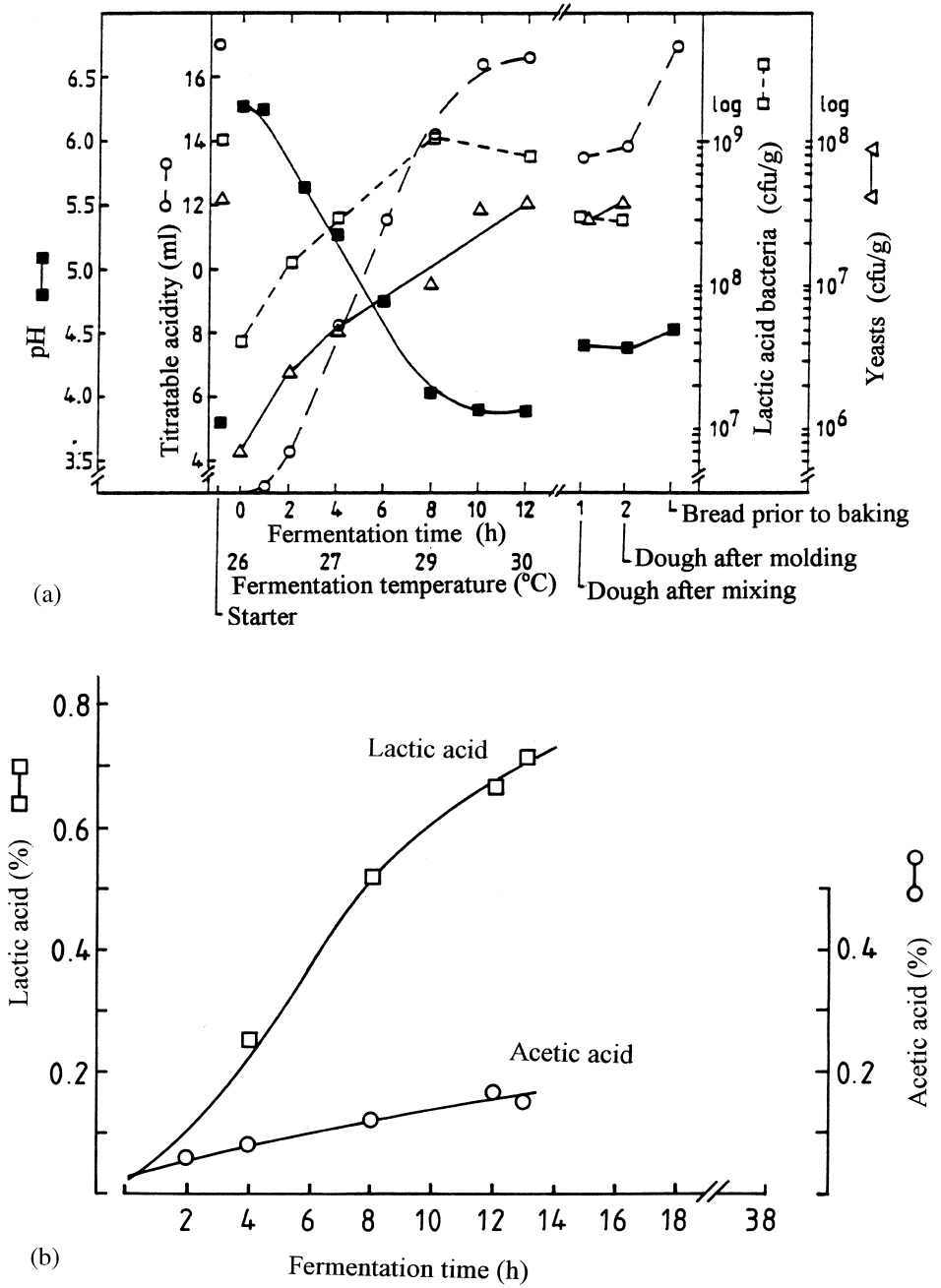

Figure 1 (a) Development of pH, total titratable acidity, and CFU of lactic acid bacteria and yeast in an industrial rye sourdough process inoculated with 3% sourdough from previous batch. (b) Development of lactic acid and acetic acid in an industrial rye sourdough during fermentation.

of wheat sourdoughs have been inspired by the symbiotic relationship found in the San Francisco French bread process and first described more than 30 years ago.[47,48]

Most food applications of lactic acid bacteria utilize homofermentative species. However, in breadmaking the heterofermentative lactobacilli also play a major role, and the acetic acid formed in heterolactic fermentation is essential to bread flavor and shelf life. Often in a given rye sourdough numerous species of lactobacilli can be detected but only one to three species are dominant and found in typical high numbers. Obligately homofermentative, facultatively homofermentative, and heterofermentative types of lactobacilli are found in sourdoughs. Precise identification of strains isolated from sourdoughs has always been problematic, partly because the properties of the isolated strains do not always fully comply with those of the model strains originating from other ecological environments. Molecular identification and clustering have recently been applied, in particular by German and Italian sourdough microbiologists.[31,49,50] *Lactobacillus* species frequently identified in sourdoughs are listed in Table 4. Application of molecular methods has deepened the understanding of heterofermentative species in particular.

Industrial sourdough processes seem to be microbiologically and functionally very consistent, indicating the presence of a highly adapted flora. Stability of sourdoughs that

Table 4 Typical Species of Lactobacilli Detected in Sourdoughs in Various Studies

Species identified with conventional methods Species	Ref.[a]	Species identified applying molecular and conventional methods	Ref.[a]
Obligately homofermentative			
L. acidophilus	[51,52,54,55]	*L. acidophilus*	[49]
L. farciminis	[51,54,59]	*L. amylovorus*	[50]
L. delbrueckii ssp. *delbrueckii*	[53,54,56,62]	*L. delbrueckii* spp. *delbrueckii*	[49]
Facultatively heterofermentative			
L. plantarum	[51–54,56,59,63]	*L. plantarum*	[49,64]
L. casei	[51–53,56]	*L. lactis* ssp. *lactis*	[49]
L. rhamnosus	[54,55]		
Obligately heterofermentative			
L. sanfranciscensis (= *L. sanfrancisco*) identical to *L. brevis* var. *lindneri*	[48,51,54–56,58–61,63]	*L. sanfranciscensis*	[64]
L. brevis	[51,53,54,56–58,61]	*L. brevis*	[49,65]
L. fermentum	[51,53–56,60,61]	*L. fermentum*	[49,65]
		L. pontis	[50]
		L. reuteri	[50,66]
		L. johnsonii	[64]
		L. fructivorans	[65]
		L. alimentarius	[49]
		L. frumenti	[67]

[a]Data from: Ref.[48–67]

are properly taken care of in bakeries also explain why commercial starter cultures consisting of one or more well-defined species or strains of lactic acid bacteria, available as freeze-dried powders or tablets, have not found a substantial market in the baking industry. However, starter cultures containing a stable mixed flora are commercially available. The most successful of these starters is probably the Böcker "Reinzucht" sourdough, which contains *Lactobacillus brevis* var. *lindneri* (*Lb. sanfranciscensis*) as the dominant species.[41,64,68] The market for dried sourdough preparations as a bread ingredient has increased. However, these bakery premixes do not necessarily contain live lactic acid bacteria.

In some specific processes a high fermentation temperature is used to control contaminating flora. Meuser[62] reported a liquid fermentation system in which fermentation by *Lactobacillus delbrückii* takes place at 48–52°C, followed by another fermentation stage at 28–32°C for yeast proliferation.

The yeast present in a sourdough is acid tolerant and typically forms a stable combination of mutual benefit with the lactobacilli. The yeast produce amino acids, vitamins, and other growth factors required by the lactic acid bacteria,[69,70] whereas the acids produced by the lactic acid bacteria suppress the growth of other microbes. *Candida milleri* appears to be the most typical yeast species in sourdoughs, although baker's yeast *Saccharomyces cerevisiae* and other closely related strains are also detected. Many of the sourdough yeast strains now designated as *C. milleri* were earlier referred to as *Torulopsis holmii*, the asporogenous form of *Saccharomyces exiguus.* There is a nice symbiosis between *C. milleri* and *Lb. sanfranciscensis* (formerly *Lb. sanfrancisco*, identical to *Lb. brevis* var. *lindneri*). This was first described for the San Francisco sourdough French bread process by Kline and Sugihara.[48] Later a corresponding relationship was detected in sourdoughs from elsewhere, as in a German sourdough starter, a Sudanese kisra sourdough, and an Italian panettone sourdough.[60,71,72]

An obvious key factor in this symbiotic and highly resistive relationship appears to be the strong preference of the lactobacilli for maltose, as reviewed by Gobetti and Corsetti[73] and Vogel et al.[44] and clarified earlier in detail by Stolz et al.,[60,74–76] Neubauer et al.,[77] Gobbetti et al.,[72,78] and Gänzle et al.[31]

In these sourdoughs the *Lb. sanfranciscensis* utilize only maltose while releasing glucose that is used by the *C. milleri* yeast, which is itself incapable of assimilating

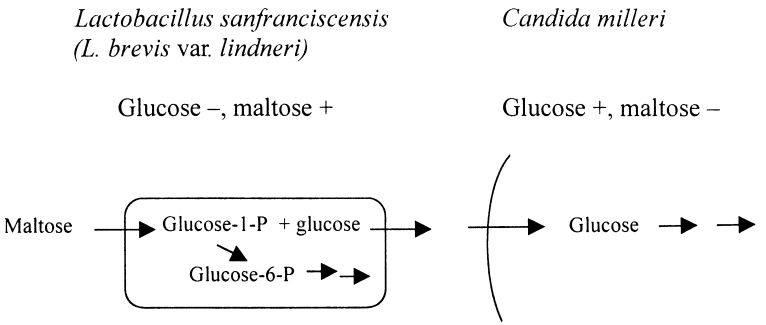

Figure 2 Utilisation of maltose in a sourdough by heterofermentative lactobacillus *L. brevis* var. *lindneri* and *Candida milleri* yeast. The lactobacilli utilize only maltose and release glucose, which is assimilated by the yeast.

maltose (Fig. 2). The lactobacilli found in these systems have the enzyme maltose phosphorylase, which is cabable of phosphorylating maltose to glucose-1-phosphate and glucose without the expenditure of ATP. Since there is plenty of maltose present in a sourdough, the lactobacilli can release glucose into the medium and use the energetically more favorable pathway to glucose-1-phosphate and further to glucose-6-phosphate via the phosphoketolase pathway (Fig. 3). In case all the maltose is used, the *Lb. sanfranciscensis* can take up the glucose that it earlier excreted. The mechanism improves the competitiveness of *Lb. sanfranciscensis* in the symbiotic microecological system, as the glucose released by the lactobacilli causes maltose repression in competitors leaving *Lb. sanfranciscensis* as the only exploiter of maltose in the system.[44,60,74–76]

Another application in which a better understanding of the biochemistry of sugar fermentation by heterofermentative lactobacilli has proved to have practical use is the

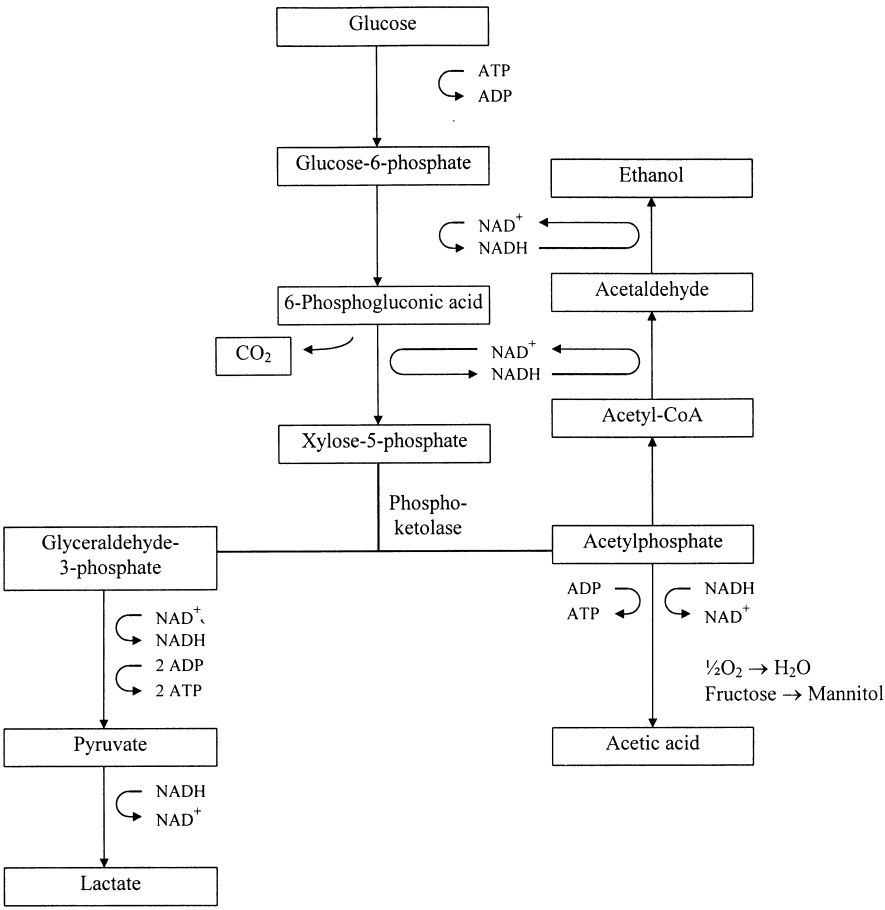

Figure 3 Fermentation of glucose in heterofermentative lactobacilli. The reaction leading to ethanol produces no ATP, but 2 mol of NAD^+ are regenerated. In the presence of an added hydrogen acceptor (fructose or molecular oxygen), acetylphosphate is converted to acetate and an additional 1 mol of ATP is formed.

understanding of acetic acid production in sourdough for improvement of mold-free time, rope prevention, and flavor (see also Table 3). Heterofermentative lactobacilli lack aldolase, a key enzyme of glycolysis. Instead, they have phosphoketolase and produce carbon dioxide and ethanol/acetate from glucose in addition to lactate.[79,80] In the reaction pathway acetylphosphate is reduced to ethanol (Fig. 4). This enables 2 mol of NAD^+ to be regenerated, but the reaction yields no ATP and so the net result is only 1 mol of ATP from glucose in comparison to the 2 mol produced by homofermenters. However, many heterofermentative lactobacilli can use molecular oxygen or fructose as an electron acceptor and regenerate NAD^+ by converting acetylphosphate to acetate instead of ethanol.[81] This reaction path produces one additional mole of ATP from glucose and explains why some heterofermentative lactobacilli grow better in the presence of oxygen than anaerobically.[79] In the reaction oxygen is reduced to water or hydrogen peroxide, whereas fructose is reduced to mannitol.[73,74,75]

It has been known for a long time that by making a hydrogen acceptor available for the heterofermentative lactobacilli, the fermentative pathway can be shifted so that acetate production is favored over ethanol.[81,82] Röcken et al.[83,84] showed that in baking applications the addition of fructose in the form of invert sugar had a linear effect on acetate content of sourdough. As shown in Fig. 4 the presence of added baker's yeast did not negate the effects of fructose on acetic acid accumulation in a rye sourdough containing a mixed flora including heterofermentative strains.[85]

In consideration of the microecological system present in sourdoughs, it can be concluded that the competitiveness of certain heterofermentative lactobacilli in sourdoughs is supported by their characteristic capability to use of both maltose and electron acceptors. In addition, *Candida milleri* is capable to degrade glucofructosans present in flour and release fructose that can be used as an electron acceptor by *Lb. sanfranciscensis*, and so the combination is strengthened again.[44] The presence of *Lb. sanfranciscensis* has been reported only in sourdoughs.

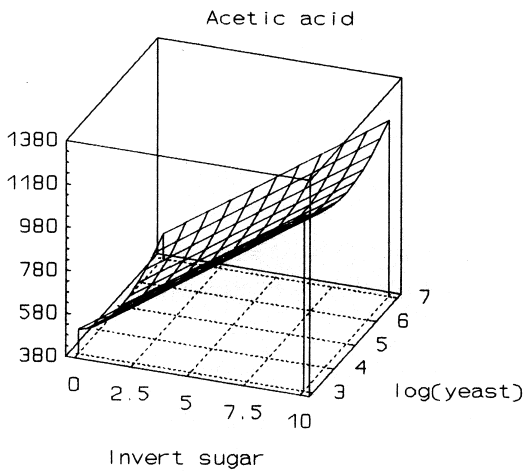

Figure 4 Concentration of acetic acid in rye sourdough (mg/100 g flour) as a function of invert sugar addition (%) and baker's yeast addition (log CFU/g flour) at 37.5°C. (From.[85])

V. TRADITIONAL LACTIC ACID–FERMENTED CEREAL-BASED FOODS OTHER THAN BREAD

From a global perspective a considerable part of the cereal-based foods made by lactic acid fermentation are products other than sour bread. Such meals are staple foods in Africa and in parts of Asia and South America. For example, it was estimated that 45% of the maize used in Cotonou in Benin was processed into fermented foods.[6]

Fermented cereal-based foods other than bread include beverages, gruels, dumplings used in stews, and fried products. Maize, sorghum, millet, and other starchy materials are used for the preparation of these indigenous cereal products.[4,20,46,48] Fermented porridges were also known in Europe either as indicated by the reports on sowens, flummeries, and other similar oat-based products.[5,9–11]

Examples of indigenous cereal-based products that have been scientifically studied are ogi and agidi (Nigeria), koko, akassa, and kenkey (Ghana), uji (East Africa), mawè (Benin), and mahewu (southern Africa). Many of the these products are also made commercially for local markets.[2,6,46]

The preparation of fermented sour cereal foods other than bread often follows the simplified pattern shown in Fig. 5. Maize, sorghum, or millet grains are soaked in clean water for 1/2–3 days. Mixed fermentations, including lactic acid fermentation, take place during the soaking stage. Soaking softens the grains and makes them easier to crush or wet-mill into a slurry, from which hulls, bran particles, and germs can be removed by screening and sieving procedures.

Slurrying in water of the material from either wet- or dry-milling supports fermentation, which is allowed to take place overnight or for longer, usually at ambient temperature. The slurrying or doughing stage has many similarities with the sourdough procedures used in traditional European sourdough breadmaking. Back-slopping, i.e., saving part of the previous batch as a starter for the next batch, may be used. The equipment used

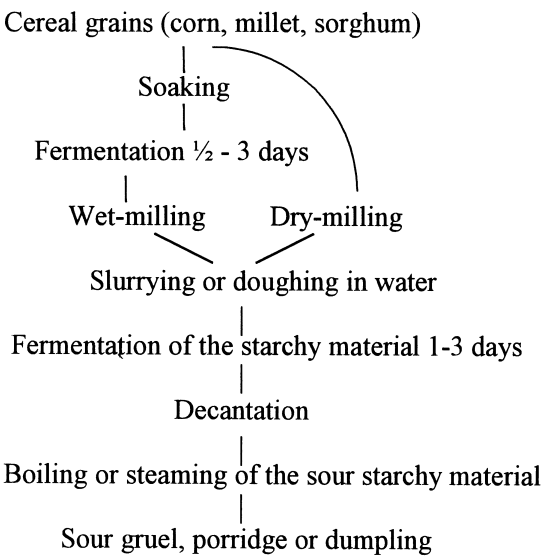

Figure 5 Simplified flow sheet of traditional sour porridge preparation.

may also serve as a source of starter organisms.[3,4,6] Accumulation of acids occurs much in the same way as in a sourdough for bread, as can be seen from the data presented by Akinrele,[86] Andah and Muller,[88] Mbugua,[89] Hounhouigan,[6] and others. In a fully fermented slurry the number of lactic acid bacteria may be as many as 10^9 CFU/g, which equals cell densities found in a fermented sourdough. When the fermentation is completed, the slurry is boiled with an appropriate amount of water so that gelatinization of starch occurs and a product of desired consistency is obtained. The final product may be drinkable, spoonable, or stiff and dumpling-like.

The microorganisms responsible for the souring process have been reported in several studies, as selectively summarized in Table 5. Features of some processes described in the literature and the role of lactic acid bacteria in them are briefly outlined below.

Ogi is a fermented semi-product made mainly from maize. It serves as an important meal for people of all ages in West Africa and has many culturally related names and modifications.[20,46] The fermentative microbial flora responsible for the pH decrease in Nigerian ogi production was dominated by *Lactobacillus ssp*.[90] Ogi is actually the name for the sour starchy sediment marketed locally by women. Various dishes are made from ogi by boiling it with water to make gels of variable stiffness. These include akassa, a stiff gel-like product eaten with fish or meat stew, agidi, a dumpling-like food wrapped in leaves, and koko, a porridge-like food used for breakfast. Like ogi, these end products are commercially available in local markets in western Africa (Adeyemi and Oluwamukomi, 1989).[2,4,6,20,86]

The preparation of kenkey differs from the general outline described above in that only part of the fermented maize dough is boiled, and the boiled fraction is mixed back into uncooked dough and allowed to ferment another for 3–8 hours. The resulting stiff paste is wrapped in leaves and the dumplings are prepared by steaming, boiling, or baking.[2,5,94,98] The main lactic acid bacteria present in the fermenting kenkey doughs were identified as *Lactobacillus plantarum*, *Lactobacillus confusus*, *Lactobacillus brevis*, and *Pediococcus pentosaceus*.[99]

Table 5 Lactic Acid Bacteria Found in Slurries for Fermented Gruels, Dumplings, and Beverages

Species	Substrate (Ref.)[a]
Lactobacillus plantarum	Ogi,[90,86] uji,[89] mageu,[92] kishk,[2] togwa[97]
Lactobacillus delbrueckii	Mageu[92]
Lactobacillus casei	Kishk[4,93]
Lactobacillus fermentum	Mawè,[95] kenkey,[96] togwa[97]
Lactobacillus reuteri	Mawè,[95,4] kenkey[96]
Lactobacillus brevis	Mawè,[95] togwa[97]
Lactobacillus cellobiosus	Mawè,[95] togwa[97]
Leuconostoc mesenteroides	Koko,[91] kenkey,[94] idli,[23] puto[4]
Leuconostoc fermenti	Koko,[91] uji,[89] kenkey[94]
Pediococcus acidilactici	Kenkey[94]
Pediococcus pentosaceus	Togwa[97]
Streptococcus lactis	Mageu[92]
Streptococcus faecalis	Idli[23]

[a]Data from: Ref.[2,4,23,86,88–97]

Mawè is another fermented maize-based semi-product, found in Benin. In contrast to the ogi process mawe is milled without a prolonged soaking stage and the resulting flour is mixed with water to form a fermenting dough, rather than a slurry.[6] Heterofermentative lactobacilli, such as *Lactobacillus fermentum*, *Lactobacillus reuteri*, and *Lb. brevis*, dominate in the fermentation of mawè.[95,96]

Uji is a fermented thin gruel or sour beverage from eastern Africa. It is made from a mixture of maize flour and millet, sorghum, or cassava flour. Sucrose is often added. Uji is one of the major foods in East African diets. The microbiology of uji has been studied by Mbugua and coworkers.[7,89]

Sequential fermentation by lactic acid bacteria and yeast is used in the opaque sorghum beer process applied industrially in South Africa.[14] In this process the souring temperature is strictly controlled to 48–50°C, preventing the growth of mesophilic organisms and favoring the growth of the thermophilic strictly homofermentative *Lactobacillus delbrueckii.* After initial souring by the lactobacilli, the process involves cooking, mashing, and straining, followed by a second fermentation stage by *Saccharomyces cerevisiae* yeast. The final product is not filtered and is still fermenting when filled in road tankers and various containers which have a vent to allow the carbon dioxide to escape. The beer is subject to microbial spoilage by mesophilic lactic acid bacteria and keeps only a few days. A similar type of Kenyan beer called busaa was described by Nout.[100]

Starter cultures for the fermentation of traditional cereal foods have been developed.[6,101] Holzapfel[4] has recently discussed the potential of starter culture technologies for small-scale fermentation in developing countries.

VI. TRADITIONAL CEREAL-BASED FOODS CONTAINING LIVE LACTIC ACID BACTERIA

Processing of cereal foods is often completed with a final boiling or baking stage, which gives the food the intended texture and makes the food more digestible and palatable. This gelatinization simultaneously kills any lactic acid bacteria present in the fermented cereal material. However, there are also processes which involve fermentation after the gelatinizing heat treatment, and hence the product contains live lactic acid bacteria.

One example of this type of traditional food is a mixture of soured milk and wheat widely consumed in Egypt and elsewhere in the Arab world. The process involves mixing fermented milk with wheat flour derived from boiled, dried, and ground wheat grains. The resulting dough is formed into balls and sun-dried to make the kishk.[20,86,102] Indian rabadi is a corresponding fresh type of product made from buttermilk and pearl millet.[103]

Lactic acid bacteria are also unavoidably present when local beers are made. Brewing of cereal grains into beer undoubtedly was an integrated alcoholic/lactic fermentation process in its original form. In industrial brewing lactic acid bacteria are normally considered undesirable contaminants. Lactic acid bacteria are in part responsible for the restricted keeping time of indigenous opaque low-alcohol beers made from various cereals in many parts of the world.[5,12,20,86] Examples of such beers are kwass, found in Russia, and opaque sorghum beers known in Africa.

Lactic acid fermentation is intentionally utilized in the industrial production of certain cereal-based sour beverage specialties. Representatives of *Lactobacillus* and *Pediococcus* belong to the fermenting flora of lambic beer made from barley and wheat, a specialty of Belgium. Typical of the process is a very long fermentation period, which takes 2 years or more. The result is a fairly strong, sour alcoholic beer sold in

bottles. There is a varying live microbial flora in the lambic beers, consisting mainly of various yeasts and lactic acid bacteria.[13,104]

In addition to the sour alcoholic sorghum beer discussed earlier, there is also a corn-based sour non-alcoholic beer, mageu (mahewu) inoculated with a *Lactobacillus* starter commercially produced in South Africa.[15,92] In its original form mageu is made by adding maize meal to boiling water and cooking for 10 minutes. Some wheat flour is added to provide amylolytic activity. A spontaneous inoculum is used, *Streptococcus lactis* being the main organism. Industrially produced mageu is made by using starter organisms such as *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, or *Lactobacillus bulgaricus*.[20,92]

In Tanzania a lactic acid–fermented sorghum or maize gruel, togwa, is used as a weaning food or as a beverage. Since togwa is not heat-treated after fermentation, it contains live lactic acid bacteria, such as *Lb. plantarum*.[97]

A. Cereal-Based Probiotic Foods

The probiotic food concept was introduced to clinical nutrition and food science in the 1980s and has been further developed.[105,106] The concept emphasizes the positive physiological effects of certain lactic acid bacteria and bifidobacteria that enter the colon as a food component. In terms of cereal-based foods, it is interesting to raise the question whether any of the the indigenous lactic-fermented cereal foods could carry strains with probiotic properties. Kingamkono et al.[18] found that fermenting togwa inhibited the growth of some enterotoxin-producing bacteria and reported that a significant reduction in the enteropathogen occurrence in rectal swabs of children under 5 years old was achieved when they were fed togwa. Such findings tend to refer to potential probiotic properties.

Vogel et al.[44] pointed out that the predominant strains present in sourdoughs and in other lactic-fermented cereal foods are closely related to or even identical to species found in the animal and human intestinal tract. Indeed, it has been found, for example, that *Lb. plantarum*, a frequent component in indigenous fermented cereal foods, is also a frequent inhabitant of human intestinal mucosa.[107,108] Furthermore, it was shown that a *Lb. plantarum* strain isolated from a sourdough can become established in human intestinal mucosa after ingestion.[109]

The number of beneficial bacteria in the gut may be increased by introducing large numbers of such bacteria in foods or in capsules, but an incorporation of suitable dietary oligosaccharides or polysaccharides may be even more effective. This is known as the prebiotic concept. Such polysaccharides must escape digestion in the upper gastrointestinal tract, and they must be soluble, hydrolyzable, and fermentable by the beneficial gut flora. This description relates to soluble dietary fiber, resistant starch, and unabsorbed sugars and oligo- and polysaccharides. Gibson and Roberfroid[110] suggested the term "colonic foods" for food components entering the colon and serving as substrates for the colon microbiota. Potential prebiotic compounds in cereals are arabinoxylan of rye and wheat and possibly β-glucan of oat and barley.[111–113]

Probiotic strains can be incorporated in cereal-based lactic fermented foods. Molin et al.[114] suggested the potential of an enzyme-treated fermented oatmeal soup as a nutritive solution for enteral feeding. Another possibility, now in commercial production, is to use cooked oat bran as a substrate for probiotic bacteria and make a yogurt-type nondairy snack, or vellie, which combines the postulated physiologically beneficial effects of oat

bran and those of probiotic bacteria and serves as an alternative to milk-based and soy-based yogurts (Salovaara et al., 1990).[1,115,116] Recent studies have confirmed the suitability of oat and other cereals as substrates for probiotic lactic acid bacteria.[108,117–120]

VII. CONCLUSIONS

Lactic acid bacteria are utilized in the production of cereal-based products in many ways. The lactic acid fermentation contributes beneficially both to processing technology and to quality of the end products in terms of flavor, keeping properties, safety, and overall image of the product. Among cereal foods most scientific research and technological development with respect to lactic acid bacteria has been associated with the sourdough breadmaking process.

Traditional fermented cereal foods other than bread, i.e., soured porridges and dumplings, have received some scientific attention. These foods have a major role for millions of people, especially in Africa and Asia, and deserve research and development input in order to improve the quality and attractiveness of these foods as economical and nutritious staples. Proper control of fermentation conditions and use of a starter, possibly from systematic back-slopping procedures, might be useful ways to give added value to the local cereal-based foods. One obvious possibility for any market is the use of selected probiotic starters for the fermentation.

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16

Human Lactic Acid Microflora and Its Role in the Welfare of the Host

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I. GENERAL INTRODUCTION

The host and its indigenous microflora together form a complex ecological system. Various exogenous and endogenous influences direct the developmental pattern and balance/imbalance of the system. In the last edition of *Lactic Acid Bacteria*, our main interest was focused on the substantial question: How is the microflora established to form a well-functioning ecological system? Neonate and experimental animals have served as useful natural models for revealing the ecological impact on the development and stability of human microflora. The various aspects of composition, maintenance, and formation of individual human lactoflora were summarized in the previous edition and are once more addressed in somewhat compressed form in the new version.

The recognition of lactobacilli, one of the most common gram-positive bacteria in the human microflora, as an important part of the human microbial ecosystem and the understanding of the various interconnected influences of that system is our starting point. Population studies and clinical investigations have underscored the large individual differences of lactoflora, in terms of numbers and variety of species, depending on the genetic background of hosts as well as their age and health.[1–8]

Further, application of advanced methods, particularly molecular genetics, into microbiological studies has significantly widened our understanding of microbial ecology in humans and experimental animals. Many observations have been attributed to the beneficial effects of indigenous microbes against pathogens.[9–12] However, the role of indigenous microflora seems to be wider than just the competitive exclusion of pathogens. There are many new advancements in the fields of innate and adaptive immunity for the protection of the host against environmental factors, e.g., pathogenic microbes. This has directed

researchers to answer another important question: What is the impact of indigenous microflora, particularly of lactic acid bacteria (LAB), on the well-being of the host?

Lactobacilli usually inhabit various organs as innocuous commensals. Since the turn of the twentieth century, human lactobacilli have been considered contributors to human health.[13] Metschnikoff's well-known statement, "There are many useful microbes, amongst which the lactic acid bacteria have an honorable place," has stimulated extensive investigation of the bacterial group's properties. The presence of lactobacilli in different areas of body and their role in host and microflora-related physiochemical conditions have become one of the subjects of microbial ecology.[1,14–23]

Recent work has brought the knowledge that the prevalence of adverse reactions to environmental factors, particularly the prevalence of allergic diseases, has increased over the past 40 years in western Europe.[24] An analysis of the possible reasons of the increased prevalence of allergy must be based on the assumption that the occurrence of factors that have the potential to stimulate allergic sensitization has increased or that hypothetical environmental allergy-protective factors have been lost.[25] The prevalence of atopic diseases correlates with the socio-economic level of a country. This tendency has been associated with the "hygiene hypothesis" and "western lifestyle."[25,26–29] It has been suggested that the western diet with its increased intake of unsaturated fatty acids and free oxygen radicals might be one triggering factor. However, microbial exposure is also a potent exogenous factor in the maturation of the immune system.[29–33] Therefore, the role of indigenous microflora in the prevention of allergic sensitization has been the attention of several investigators[34–36]

In this chapter we summarize studies[37,38] concerning differences in the composition of intestinal microflora during neonatal period and infancy in Estonia with a low and Sweden with a high prevalence of childhood allergy.[39,40] These studies confirm the data of previous authors regarding the existence of geographical differences in the human microflora.[41,42] Moreover, cross-sectional and prospective studies have shown pronounced differences in the establishment of intestinal microbes in allergic as compared to nonallergic children.[43,44] These findings have encouraged the hypothesis regarding the role of particular intestinal microbes in the induction of oral tolerance, allergic sensitization or downregulation of mast cells reactions to allergens at the different periods of life (neonate, infant, 1- to 5-year-olds).

The achievements in human microbial ecology, immunology, and nutrition as well as a better understanding of the pathogenesis of chronic cardiac and neurological diseases have been the basis to start new prophylactic measures for human health. New trends such as functional foods have started to influence large segments of the population.[45] Probiotics, mainly lactic acid bacteria, are considered the part of functional food with potential health-promoting traits for prevention of intestinal tract infections and/or stimulation of several host physiological factors.[46] Among probiotic lactobacilli, different functional capabilities have been claimed for certain strains including antiatherogenic, anticarcinogenic, anticonstipation, and antiallergic potential.[47] Another important aspect is health promotion starting from birth. The possibility of controlling the colonization of neonates could open new perspectives for prevention of infections and allergy.

This chapter introduces a novel probiotic strain *Lactobacillus fermentum* ME-3 (DSM 14241) expressing substantial antimicrobial and antioxidative effects.[48] These health-promotional properties of the strain are promising and in addition to improvement of gut microbial interrelations, an impact on the biotopes antioxidative conditions has been suggested. Recently, in an experimental mouse model of *Salmonella* Typhimurium infection, we demonstrated the possibility of balancing the antioxidativity of damaged gut

mucosa during the oral administration of *L. fermentum* ME-3.[49] This is the basis for understanding how the strain ME-3 can exert the antioxidative and antiatherogenic effects, as shown recently in blood sera of volunteers.[50]

II. LACTOBACILLI IN THE HUMAN MICROBIAL ECOSYSTEM

A. Components of the Microbial Ecosystem

On their surfaces and organs, humans harbor a normal, or indigenous, microflora,[51,52] the number of which ($10^{13}-10^{14}$) exceeds the total number of body cells 10 times.[53] These populations of microorganisms form open ecosystems in humans featuring dynamic, yet relatively stable interactions between the normal microflora of various locations and the host.[54–56] The human gastrointestinal, genital, and skin microbial ecosystems are the most diverse, harboring the highest populations of microorganisms. Ducluzeau[56] defined an ecosystem as comprising a group of microbial populations coexisting in an equilibrium in a spatiotemporally defined region. From an ecological point of view, the inclusion of terms such as microbiocenosis and microbiotope could be helpful in describing human microbiota.

The main unit of a microbial ecosystem is the microbiocenosis: the association of qualitatively different groups of microorganisms having dynamically fluctuating but relatively stable quantitative characteristics.[16,57,58] A microbial ecosystem always contains several microbiocenoses located in certain microbial biotopes of the host.

A microbial biotope (microbiotope) is an area or place suitable for the survival and growth of particular microorganisms.[16] The gastrointestinal mucosal surface is calculated to be 150–200 m^2.[59] More than 75% of the wet weight of the fecal output is composed of bacteria, with each gram containing approximately 1×10^{11} microbes.[60] There exists a vertical distribution of microorganisms in the gastrointestinal tract, differentiating between gastric, duodenal, jejunal, ileal, cecal, colonic, rectal, and fecal microflora. One of the most important scientific achievements in human microbial ecology studies has been the discovery of an association of microflora with definite habitats and its division into luminal and mucosal for different organs.[61–65] Zoetendal et al.[66] showed that in the colon, denatured gradient gel electrophoresis (DGGE) profiles of predominant populations were highly similar in different individuals, while they differed significantly from those of fecal populations. This indicates that different populations of microorganisms predominate in the colonic mucosa and the feces.[67] These findings also explain why the complex microbial communities of different biotopes are comprised of both transient and relatively persistent components.[68] Analogous divisions could also be found in other microbial ecosystems, e.g., the urogenital niche.[69] The microbial biotope determines not only the anatomical localization for the microbiocenosis but also its functional state through a complex of host- and microflora-derived physicochemical conditions, including pH, redox potential, nutrient availability, peristalsis, and transit time.[70–72]

Recent studies have indicated that bacteria that reside in the GI tract are far from inert commensals, but are actively communicating with the immune system and gut epithelial cells.[73] This can result in clear protection against a wide range of mucosal pathogens and influence host welfare.

1. Systematics of *Lactobacillus* spp.

Lactobacilli are gram-positive, rod-shaped, facultatively anaerobic, nonsporulating, acid-tolerant, and catalase-negative bacteria with a DNA base composition of less than 53 mol% G + C. The grouping of lactobacilli has developed from division into the subgenera like

Thermobacterium, *Streptobacterium*, and *Betabacterium* according to their growth temperatures and their hexose fermentation pathways.[1,74–76] However, it is widely acknowledged that the taxonomy of the *Lactobacillus* genus is unsatisfactory due to phenotypic heterogeneity.[77] Modern molecular methods have shown that these subgroups are inconsistent with the phylogenetic relationship of the species within the genus.[78] This is the case particularly for the *Lactobacillus acidophilus* and *Lactobacillus casei* groups.[79] Using classical phenotypic characterization, the *L. acidophilus* group may be divided into different biotypes, which again do not correspond with the various phylogenetically defined species within the genus.[80]

Despite the fact that the general basis for the classification of LAB in different genera has largely remained unchanged since the work of Orla-Jensen,[81] phylogenetic classification now relies on molecular biology methods, especially on the species level. The identification and phylogeny of bacteria has been based on the comparison of highly conserved molecules of the 16S ribosomal ribonucleic acid (16S rRNA) genes.[82] The studies based on 16S rRNA sequences have shown close relationships between the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Oenococcus*, and *Weissella*. The principal groupings are summarized as follows: (a) the *Lactobacillus delbrueckii* group, (b) the *L. casei–Pediococcus* group, and (c) the *Leuconostoc* group, including species from the genera *Lactobacillus*, *Ocenococcus*, and *Weissella*.[83] In addition, based on the type of cell wall and the fermentation pathways for pentoses and hexoses, lactobacilli are divided into three groups as follows: obligately homofermentative lactobacilli (OHOL), facultatively heterofermentative lactobacilli (FHEL), and obligately heterofermentative lactobacilli (OHEL).[84]

Lactobacilli can be identified by means of gas chromatography (GC), high-pressure liquid-gas chromatography, fermentation of carbohydrates, soluble protein content, cell wall components, and DNA profile.[65] Previously, identification has been based on the phenotypic properties of lactobacilli. However, the taxonomy of the genus *Lactobacillus* is unsatisfactory due to phenotypic heterogeneity.[77] Therefore, the classical culture-based techniques are now being supplemented with molecular tools. Recently, molecular biological methods such as polymerase chain reaction (PCR) with specific primers, DGGE (Fig. 1), and temperature gradient gel electrophoresis (TGGE) have been shown to be powerful approaches in determining and monitoring bacterial community in the gastrointestinal tract.[85–87]

B. Lactobacilli in Various Biotopes

Human microflora consists of a large number of different groups and species of microorganisms. The human body is inhabited by more than 500 different species, among them lactobacilli.[88,89] Interest in their occurrence, numbers, and role within microbial ecosystem of humans has survived a whole century.[1,13–15,21,90–94] Lactobacilli have been found in the gastrointestinal tract, vagina, on the skin, in the nasal and conjunctival secretions, and in the ear, breast milk, and sperm.[87,95–98]

1. The Digestive Tract

Sufficient data are available regarding *Lactobacillus* spp. in the proximal and distal parts of the intestine, but knowledge about the content of lactobacilli in the ileum, cecum, and colon of healthy subjects is inadequate (Fig. 2). Therefore, our present knowledge on the bacterial diversity of the gastrointestinal tract is based mainly on analysis of oral and fecal

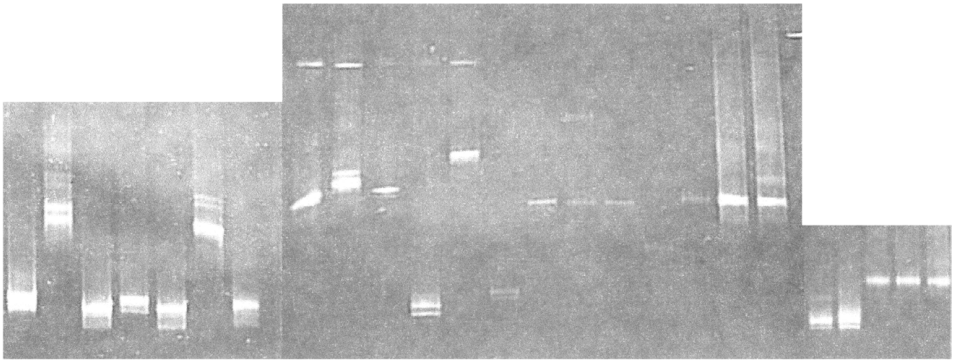

Figure 1 Species-specific identification of lactobacilli by denaturating gradient gel electrophoresis (DGGE) method. (A) Identification of representatives from FHEL group using primer set ENV1 and TTGE-gc (amplification between U1 and U2 regions of the 16S ribosomal RNA gene). (B) Identification of representatives from OHOL and OHEL group using primer set Lac1 and Lac2-gc (amplification of the V3 region of the 16S ribosomal RNA gene). (C) Differentiation between representatives from OHEL group (L. fermentum and L. reuteri) using primer set Lac1 and Lac2-GC (amplification of the V3 region of the 16S ribosomal RNA gene).

samples. The same can be said about the mucosal lactoflora, and the main reasons for this are the methodological limitations of obtaining biopsy samples from these areas.[65,67,70,99]

Oral lactoflora has been investigated in numerous studies. Lactobacilli are common inhabitants of the oral cavity, although they usually comprise less than 1% of the total cultivable microflora.[100,101]

	LUMEN	MUCOSA
MOUTH	**8 ... 9.0**	
STOMACH	**<3.0**	**>3.0**
DUODENUM	**<=3.0**	
	<4.0	**3.1 ... 4.6**
JEJUNUM	**2.1 ... 4.3**	**3.8 ... 4.1**
ILEUM		
CAECUM	**7.4**	
COLON	**<5.0**	
	3.0…7.0	
RECTUM	**4.0 ... 8.0**	
	0 ... 10.0	**0 ... 6.3**
	(70%)	**(50%)**
FECES	**6.0... 10.0**	

Figure 2 Schematic representation of lactobacilli numbers (log CFU/g) in a healthy human's gastrointestinal tract. [Data adapted from[6,67,68,89,96,101,106,108,121,122].]

The microflora of the small intestine has been regarded as a mixture of gastric and colonic microflora, the latter appearing mainly in the ileum. The numbers of microorganisms in the proximal jejunum are approximately 10^4/mL while the oropharyngeal microflora is predominating. However, in a recent study *Lactobacillus* microorganisms were revealed in only 2 of 20 healthy subjects.[102] Lõivukene et al.[103] showed that rare colonization by *Lactobacillus* spp. was also characteristic of the gastric mucosa of *Helicobacter pylori*–negative children (9/65) and adults (1/29).

In recent fecal studies, a 60–80% prevalence of lactobacilli was detected in one-month-old infants.[104] In adults it was found that *Lactobacillus* strains were present in the gastrointestinal tract of approximately 70% of humans that consume a western-type diet.[8] In elderly persons the prevalence of lactobacilli is high.[96,105]

Earlier studies demonstrated that in the fecal microflora of adults the *Lactobacillus* spp. counts reach 10^{10} CFU/g and are outnumbered only by obligate anaerobes.[89] However, more recent studies have shown that facultatively anaerobic lactobacilli did not usually exist at such high counts in feces, reaching only 10^8–10^9 CFU/g in children.[37] It is possible that the 10-fold higher counts were previously obtained by grouping lactobacilli together with bifidobacteria.

We have developed criteria for the evaluation of the quantitative composition of fecal microflora. Of all fecal microorganisms, individual types of anarobes (bifidobacteria, eubacteria, anaerobic cocci, and/or bacteroids) in the predominant flora repress the subdominant flora, e.g., potentially pathogenic microorganisms (enterobacteria, staphylococci, streptococci, enterococci, clostridia, and yeasts). The following criteria for the subdominant flora were assessed (Table 1): for 5-month- to 1-year-old infants, <15%; for adults, <10% for the total microbe count.[106–108] Application of a special computer program helps to calculate the distributions of different groups of bacteria in

Table 1 Criteria for Evaluating Fecal Microflora of Infants (5 months to 1 y) and Adults (16–50 y)

	Infants			Adults		
	Range	Median	% of total count	Range	Median	% of total count
Microbes	log CFU/g		norm	log CFU/g		norm
Coliforms	6.6–9.8	8.3	<10.0	5.8–9.6	7.3	<3.5
Proteus	0–6.3	4.0	<0.02	0–6.3	3.0	<0.01
Staphylococci	0–8.3	5.0	<0.2	0–8.6	3.6	<0.01
Streptococci	0–9.4	7.8	<3.0	0–9.3	7.0	<2.0
Lactobacilli	0–9.0	6.6	<1.3	0–9.0	7.0	<0.1
Eubacteria	0–11.0	9.0	55.6[a]	0–11.0	9.0	56.3[a]
Bifidobacteria	0–11.8	9.0	99.6[a]	0–11.3	9.6	78.0[a]
Clostridia	0–7.6	5.0	<0.01	0–7.2	4.0	<0.1
Anaerobic cocci	0–11.1	10.0	94.7[a]	0–11.3	10.0	95.1[a]
Bacteroides	0–11.3	9.8	95.1[a]	0–11.3	10.0	97.8[a]
Yeasts	0–9.0	4.7	<0.1	0–6.8	0	<0.01
Total % of subordinate microbes			<15.0			<10.0

[a]Potentially predominant microbes.

the total bacteria count from a particular sample.[109] By these indices the imbalance and the shifts of the quantitative composition of fecal sample could be easily evaluated in association with different environmental factors[37,38,43,44] (Table 1). Using this approach,[106] the relative abundance of lactobacilli in the total count of fecal bacteria for particular persons has been estimated: $<2\%$ in children up to 1 year and $<0.1\%$ in adults.[108]

Sghir et al.[110] has also shown that *Lactobacillus* spp. constitute $<1\%$ of the total bacterial community within the human fecal microbiota. Recently, using a fluorescent in situ molecular hybridization technique (FISH), Marteau et al.[67] found that though the counts of cecal lactobacilli are quite close to counts of fecal lactobacilli (8.4 vs. 8.8, respectively), their distribution in total count is quite different (23% vs. 7%). These data on the high distribution levels of the *Lactobacillus-Enterococccus* group indicate their importance in the mucosal flora of the large intestine.

The lactoflora of the human gastrointestinal (GI) system consists of various species, subspecies, and biotypes of homo- and heterofermentative LAB. The most frequently occurring lactobacilli belong to six species: *L. acidophilus*, *L. salivarius*, *L casei*, *L. plantarum*, *L. fermentum*, and *L. brevis* in various combinations.[3,5,70,91,92,111,113] In addition to these, the frequent occurrence of *L. reuteri* in the GI tract of humans and animals has also been shown.[76,114]

In the oral cavity a wide range of species have been found: *L. acidophilus*, *L. salivarius*, *L. casei*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *L. cellobiosus*, *L. buchneri*, and *L. brevis*.[101,115–118] No special species or subspecies of *Lactobacillus* characteristic of the oral cavity have been assessed to date. In a recent study we isolated salivary lactobacilli from all studied persons, with most persons having high or very high bacterial counts.[119] We isolated nine species of lactobacilli belonging to all fermentation groups, the most prevalent of which were *L. fermentum*, *L. plantarum*, *L. casei*, *L. gasseri*, and *L. salivarius*.

In rectal biopsies the most prevalent colonizers of the *Lactobacillus* spp. were the *L. acidophilus* group, *L. salivarius*, *L. casei*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *L. buchneri*, and *L. reuteri*.[115,120] Zoetendal et al.[66] showed that *L. gasseri* was found in descending colon biopsy samples of 9 out of 10 adults, which indicates that this species may also be regarded as a general mucosa-associated bacterium in humans. Previously *L. gasseri* was included in the *L. acidophilus* group.

In various GI biotopes of a particular host the species of lactoflora can differ.[99] Clear differences in the composition of lactobacilli species were present in the lactoflora of saliva and feces from healthy young men.[4] The fecal lactoflora was usually richer in the number of species and biovariants. Yet the presence of different biovariants detected in saliva and feces of investigated persons coincided to some extent ($p < 0.05$, Fischer's exact test). The similarity of mucosal and luminal *Lactobacillus* spp. has been indicated by Zoetendal et al.,[66] who found that the DGGE profiles of colonic biopsy and fecal samples in close proximity to each other were similar for the *Lactobacillus* group.

2. The Reproductive Tract

The reproductive tract of healthy women contains microorganisms in the vagina and cervix, whereas the upper tract is considered sterile.[123]

The first extensive study of vaginal microflora was published in 1892 by Döderlein, who inspected stained smears and found that the healthy vagina harbored principally a

single species, named Döderlein's bacillus, today known as *Lactobacillus*. Soon the cultures of vaginal secretions revealed many other micro-organisms. The predominant microorganisms of the vagina are microacrobic and facultatively anaerobic lactobacilli, streptococci, and some other lactic acid–producing microbes.[98,124–126] From recent studies up to 100 different species have been identified in the vagina. However, lactobacilli are still considered the predominant microorganisms in the healthy vagina, their number reaching 10^8–10^9 CFU/mL.[127,128]

In early studies, *L. acidophilus* and *L. fermentum* were considered the most prevalent species of vaginal lactobacilli.[1,92] In contrast, recent studies using precise molecular methods have suggested *L. crispatus*, *L. jensenii*, and *L. gasseri* [98,129] to be the predominant lactobacilli in the vagina. Some novel species like *L. iners* [98,130] and rare colonizers of the intestinal tract like *L. jensenii* and *L. rogosae*,[131] *L. delbrueckii* subsp. *delbrueckii* and *L. agilis* [132] have also frequently been found. For example, Horowitz et al.[133] identified eight different species from the vagina: *L. acidophilus* (37%), *L. minutus* (3%), *L. jensenii* (10%), *L. fermentum* (7%), *L. casei* (24%), *L. helveticus* (3%), *L. brevis* (3%), and *L. plantarum* (6%).

The number of different species in individual samples also varies greatly. Redondo-Lopez et al.[123] found that there could be up to four species of lactobacilli in one sample of a healthy nonpregnant individual, each woman having an individual type of species composition. In contrast, in the study of Vasquez et al.[98] the vaginal lactoflora of most women consisted of one species only.

Thus, different investigators have found different species of lactobacilli in the vagina; no single species has been consistently isolated from all women. The main reasons for these varied results seem to be different sampling sites within the vagina (upper-lower part, lumen-mucosa, etc.), different laboratory techniques, and differences in external factors controlling microflora at sampling time; geographic, ethnic, and individual differences are also possible.

C. Stability of Human Microflora

1. Individuality of Microflora

The individual differences in species composition and the numbers of microorganisms in the vagina[1,17,92,98,134,137,138] and intestine[136–139] have been documented by many investigators studying the microbial ecosystems of adult organisms. However, controversial data have been obtained regarding the stability of individual microflora of various biotopes.

Earlier bacteriological studies showed that the composition of indigenous microflora varied greatly over a period of several months[140] or even as little as a few days.[141] However, the classic study by Holdeman et al.[136] found the quantitative composition of fecal microflora to be very specific for a particular host.

First, the stability of the quantitative composition of the fecal microflora of a particular host has meant the persistence of stable quantitative relations between the most common and predominant groups of microorganisms.[137,142] Similarly, after a year's study of the fecal microflora from 10 healthy volunteers, stable relationships between the numbers of different aerobic and anaerobic groups of microorganisms were ascertained as characteristic for a particular individual.[6,143]

In addition, particular strains have been found to persist over long periods. Only a few species of microorganisms inhabiting the GI tract have been followed for their persist-

ence in the same person during long periods. The stable occurrence of the same strain[8,23] or particular biotypes of *Bifidobacterium* [144] and *Bacteroides* [145,146] have been proven. Similarly, recent studies using 16S rRNA–based approaches have shown that the predominant human intestinal microflora is relatively stable from the perspective of bacterial genera and each individual harbors a specific bacterial community for at least 6 months.[8,86,147]

Furthermore, the stability of excreted bacterial metabolites has been shown to reflect the stable microbial community in the host. The microbial ecosystem as a whole is successfully characterized by biochemical studies determining the metabolites of microorganisms excreted from the human body. The very specific and stable composition of various bacterial metabolites excreted in the urine or feces has been revealed by several researchers.[71,148–156]

Thus, it is obvious that the host- and microflora-derived physicochemical conditions of microbial biotopes cannot be too similar for different persons and in that sense microbial ecosystems are always deeply individual, showing specific inter-individual peculiarities.

2. Individual Stability of Lactoflora

Feces. The number of lactobacilli in fecal samples of different persons seems to be quite variable.[99,137,157] In a survey over a 15-year period of 10 healthy volunteers[6,112,143] the ranges of counts for lactobacilli varied for each individual (interindividual variation from 5.1 to 8.3 log CFU/g). However, a stable and characteristic number of lactobacilli and the stable persistence of fecal *Lactobacillus* species were revealed for each person.[105,112] In every adult volunteer one or two species or even the same biotype occurred repeatedly, although during the study the persons aged and thus had several health failures and used some medicines. It is notable that all 10 volunteers are still in full health even now, more than 35 years later.

Similar results were obtained by studying the seasonal variation of fecal lactoflora: in 7- to 12-year-old children the same biotypes of lactobacilli were detected in 70% of cases during one-year follow-up.[158] The prolonged biological isolation of healthy persons during special training or space flight caused shifts in the numbers of opportunistic microorganisms.[159] Yet, close physical contact did not eliminate the individual specificity of their lactoflora in terms of species or bacterial number.[2,3,160] Using molecular methods (PFGE, TGGE), the stability of lactoflora on the strain level has been followed. Kimura et al.[23] showed by PFGE that eight of the nine subjects had transient strains of lactobacilli as resident intestinal bacteria for three to nine months.

This demonstrates the remarkable stability of lactoflora despite the temporary and mutable environmental conditions of the intestine of either exogenous or endogenous origin. In addition to stability, it has also been shown that several factors may influence the composition of the microflora of a normal individual. As reviewed by Moreau and Gaboriau-Routhiau,[161] in addition to differences in microflora according to age (infants and elderly persons) and diet, other factors such as stress or antibiotic treatment induce variations in human intestinal bacterial microflora, resulting in subdominant species or even pathogens becoming temporarily dominant.

There are very few studies characterizing *Lactobacillus* spp. in older people, whereas thousands of investigations have been made of newborn or infant lactoflora. Lerche and Reuter[91] could not isolate lactobacilli in the feces of elderly individuals,

but Speck[162] described higher counts of lactobacilli and clostridia and lower numbers of bifidobacteria in aging persons compared to those seen in younger adults.

In the 1970s we investigated healthy seniors (65–89 years) and found that all persons were colonized with lactobacilli. Many of these individuals had high counts of up to 8.0 log CFU/g (Table 2). Similar results (up to 7.8 log CFU/g) were shown by another recent study.[102] We further demonstrated that the species distribution of *Lactobacillus* was similar in senior and healthy adults: even the number (one to four species) of different lactobacilli species isolated from one sample coincided in both groups.[105,143]

Silvi et al.[163] have described the specific profile of *Lactobacillus* spp. in the elderly and showed a high prevalence of *L. fermentum*, similar to our study. The authors suggested *L. fermentum* as a good candidate to be utilized for the design of appropriate functional foods to fortify the intestinal microflora of the elderly. This has not been described concerning bifidobacteria and bacteroides, as the former diversity decreased and that of the latter increased in the feces of healthy elderly persons.[164,165] In elderly persons the revealed differences in *Bifidobacterium* spp. distribution have been explained by the loss of some properties of intestinal mucus necessary for adhesion of endogenous bifidobacteria.[164]

In adults, geographical differences in the composition of normal microflora appear to be mainly connected with diet, which plays an important role in the composition of intestinal lactoflora;[166] (reviewed in Ref.[72]). There are some studies comparing the microflora of adult people living in England and America with that of the people in Uganda, Japan, and India. The English people had significantly more bifidobacteria and bacteroides, but fewer LAB such as streptococci, enterococci, and lactobacilli, than Ugandans.[167] It has been concluded that vegetarian diets support the aerobic and microaerobic components of intestinal microflora.

Vagina. Premenarcheal and postmenopausal microflora are simpler and less prone to change than that found during the menstrual stage.[123,127] During menstrual flow, nutrients such as cellular constituents, hemin, and other blood proteins become a part of the total environment, the redox potential decreases, and the pH increases to 5.5–6.0. The total number of microbes decreases and the populations of lactobacilli decline concomitantly with an increase in the numbers of the other gram-positive organisms. The

Table 2 Diversity of *Lactobacillus* spp. in Healthy Young (21–44 y) and Elderly (65–89 y) People

		Young individuals $n = 10$, 43 isolates	Elderly individuals $n = 12$, 44 isolates
Homofermentative lactobacilli	*L. acidophilus* *L. salivarius*	7/10	4/12
Facultatively heterofermentative lactobacilli	*L. casei var. rhamnosus* *L. casei var. alactosus* *L. plantarum*	6/10	7/12
Obligately heterofermentative lactobacilli	*L. buchneri* *L. brevis* *L. fermentum*	8/10	7/12
		6 species	7 species

decrease of the resident *Lactobacillus* population may be related to the temporary loss of cell surface receptors during menstruation. Despite these major recurrent changes, studies show that after menstrual flow has stopped, the changes in microflora disappear so that for each woman the phenotypes appear to be stable over an extended time frame.[127,168,169]

Recent data show that transient changes in vaginal flora are much more common than anticipated. Schwebke et al.[170] performed an interesting study following vaginal flora daily in 51 women during 6 weeks. This study revealed that 78% of women have significant, although transient, changes in vaginal microflora, while only 22% of women have a normal *Lactobacillus*-dominated flora at any given time. Factors significantly associated with shifts in vaginal microflora included menses, use of vaginal medication and spermicide, condom use, number of sexual partners, and frequency of vaginal intercourse per month.

Investigations of vaginal microflora during pregnancy[124,171,172] have shown that anaerobic species decline and lactobacilli become increasingly predominant. Yeasts can also be found more frequently. Possible mechanisms of these changes include the altered hormonal state, increased pH, and increased glycogen content of the vaginal epithelium.[173] Unfortunately, few studies of the vaginal microflora have included a control group of nonpregnant women.

We have compared the vaginal microflora of healthy pregnant women with women with a threatened abortion.[134] The relative amount of lactobacilli in vaginal microflora increased as gestation advanced (Fig. 3). This shift started in women with a threatened abortion somewhat earlier (17–22 weeks of pregnancy) than in the control group (24–26 weeks). This could be explained by the influence of hormonal preparations administered to support the gravidity in cases of threatened abortion. The predominance of lactobacilli at the end of pregnancy may be considered a preventive mechanism offering protection to the fetus and neonate. This study allowed us to find seven individual types of vaginal microflora on the basis of stable predominant microorganisms. The lactobacilli-containing types (four variations) were the most common in both groups, yet the pure *Lactobacillus* type was found in only three women.[174] The microbes of greater pathogenic potential such as coliforms, clostridia, bacteroides, and β-hemolytic streptococci, did not form a stable part of the microflora. Some types (coryneforms and cocci) were strongly correlated ($r = 0.67, p < 0.01$) with bacterial vaginosis, which was found in nearly one third of the samples. This finding corresponds to the study of Onderdonk and Wissemann,[127] who posited that no one type of vaginal microflora is "normal" for all women.

Vaginal microflora has been shown to be self-regulating according to some external influences. It has been revealed that parenteral short courses of penicillin or cefazolin do not alter the vaginal flora.[175] At the same time, Hooton et al.[176] found that spermicide caused a decrease in vaginal *Lactobacillus* counts. Unfortunately, they did not follow up the vaginal flora of these women. In women exposed daily for 7 days to a douche product containing chlorhexidine gluconate 0.5%, one week after the last application of the douche, the vaginal flora was virtually identical to that observed before the course. A similar study with povidone iodine also showed stability of the flora.[177] The data of Ness et al.[178] indicate the self-regulating nature of vaginal flora: douching at least once per month was related to increased frequency of bacterial vaginosis, and recent douching (within 7 days before examination) posed the greatest risk for bacterial vaginosis. Preventive douching with antiseptics may temporarily destroy the normal flora and its colonization resistance and keep the pathogens alive.

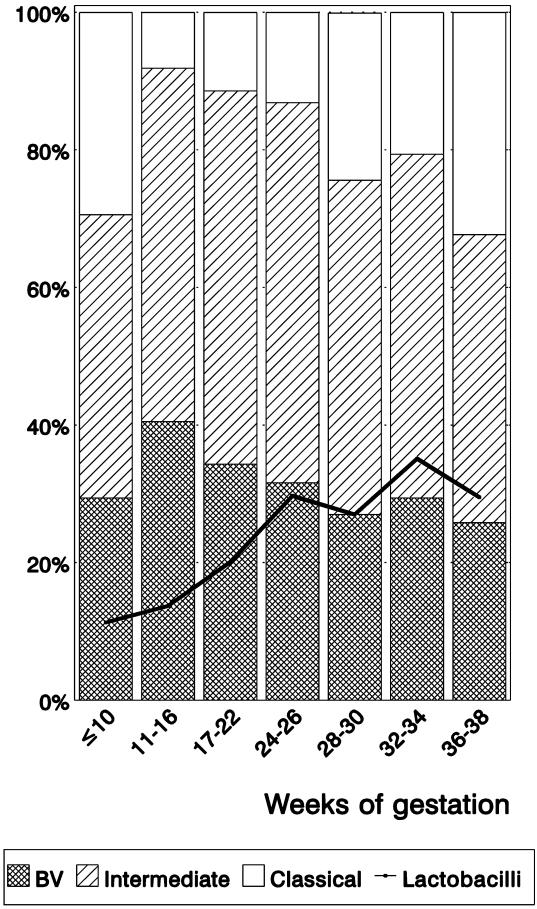

Figure 3 Dynamics of the relative proportion of lactobacilli and occurrence of bacterial vaginosis (BV) during pregnancy.

Stability of the vaginal flora emphasizes the point that microorganisms colonizing mucosa are well adapted to their environment, and even when perturbation of the ecosystem occurs recolonization is likely. The problem of individual stability of human microflora is closely connected to its formation, mainly during vaginal delivery and contamination with the mother's vaginal, perineal, and intestinal microbes.

III. ESTABLISHMENT OF INFANT MICROFLORA

The pathways, mechanisms, and factors that play a role in the contamination of the sterile fetus during labor and of the newborn during the first hours and days of life, also determining its microflora formation, have attracted the attention of scientists for nearly 100 years.[104,150,179–183]

Two stages can be distinguished in the formation of normal human microflora: first, the aquisition of microorganisms by transmission of the mother's microorganisms and, second, the successive colonization of the different habitats of the neonate by bacteria

of different environmental origins.[51,184] Real colonization means the persistence of microorganisms in the biotope up to 14 days after first appearance.[185,186]

A. Transmission of Microorganisms from Mother to Infant

Several authors suggest that the neonate is sterile during intrauterine life.[95,187] Contamination with commensal bacteria, derived from the microflora of mother's vagina, intestine, and skin and from the environment, occurs soon after birth.[188–191] Many of these microbes are unable to colonize habitats in the neonate and disappear soon after birth, whereas other microorganisms persist or may support successive colonization during the early life period to form climax communities in the adult.[52,63]

The interest of investigators in the above field has been focused in two directions. The practical goal of preventing antenatal and perinatal infections, particularly early sepsis, has been the predominant area. The main etiological agents are group B streptococci (GBS), enterobacteria, *Listeria*, *Haemophilus influenzae*, *Clostridium perfringens*, and coagulase-negative staphylococci (CONS).[183] A newborn obtains these bacteria from the mother's urinary, genital, or intestinal tract. Some 40–60% of pregnant women have been colonized by virulent, encapsulated CONS and some 4–31% of them by GBS.[182] However, due to the transplacentally acquired antibodies, of the 50% of neonates who become contaminated with mother's GBS, only 1–2% present with clinical symptoms.[192] Enterobacteria are considered one of the most frequent agents of neonatal infections. Usually the more virulent strains of *Escherichia coli* persist in the intestinal tract of the mother. The innate resistance of the neonate seems to be crucial in the prevention of the development of early sepsis.

Opportunistic microorganisms are kept in mutual or commensal interactions with the host by various factors of the macroorganism and other microorganisms of the indigenous microflora.[51,193] Resident strains of the indigenous microflora, through various mechanisms, protect the baby from the very beginning of its life from randomly acquired opportunistic strains.[95,187,190,194]

Thus, the second area of the investigations of microflora transmission from mothers to newborns has concentrated on the problem of how the colonization-controlling indigenous microflora is formed. In this respect there has been a resurgence of interest in the colonization of the neonate by lactobacilli.[38,97,182,196]

1. Early Contamination of Newborn

Several studies have shown that the gut of neonates is quickly colonized by facultative anaerobes, including enterobacteria, staphylococci, lactobacilli, and streptococci.[183,191,197,198] This is followed by colonization with anaerobic genera such as *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, and *Peptostreptococcus*.[183,190,191,198,199,200–202]

In the initial phase, the intestinal microflora depends on the mode of delivery as well as on several maternal and environmental variables. Several studies have described the early contamination of the newborn, comparing the neonate's and mother's microflora. Data on the transmission of lactobacilli from mother to neonate during birth, mode of delivery, hospital conditions, and the effect of feeding on the establishment of lactoflora in economically diverse countries contribute to the understanding of the influence of various environmental factors on neonatal lactic acid bacteria.[125,135,182,203–207]

Rotimi and Duerden[189] in their now classic study found that immediately after birth lactobacilli were present both in children's mouths and in their mothers' vaginas in 52% of cases. Only in rare cases were lactobacilli isolated either from the mother (14%) or from her neonate (9%). The presence of lactobacilli in human milk and maternal skin and their stable persistence in cecum and feces[67] surely facilitates the transfer of the aforementioned microbes from mother to neonate, as demonstrated by several studies.[95,97,125]

We have performed two studies comparing the delivery tract of 21 mothers before any treatment with antiseptics and their newborns' external ear canal microflora immediately after birth. The material from the neonate's external ear canal was considered representative of the transfer of microorganisms from mother to the neonate because this area seems to be rarely contaminated by the hands of the obstetrician. The most common microorganisms were the same for both the mothers and their newborns: lactobacilli, epidermic staphylococci, and nonhemolytic streptococci. Various individually different combinations were observed in mothers and their babies. All of the microorganisms detected in the ear of a child were present in the samples taken from its mother's perineum, but not all mothers' microbes were found in their newborns.

In the second study, 19 mother-newborn pairs were examined immediately after delivery.[125] For the newborns, the specimens were obtained from the external ear canal as in the previous study; for the mothers, high vaginal swabs were taken. Before delivery, at least two vaginal and perineal douching applications with a chlorhexidine solution (0.005%) were performed. We isolated 16 different groups of microorganisms from mothers and 13 from newborns, with the most common microorganisms being streptococci. Various individually different combinations of up to eight microbes were observed per sample in mothers and up to six microbes per sample in babies. As many as 84% of mothers and 74% of newborns were heavily colonized, and all minimally colonized mothers delivered minimally colonized babies. Bonang et al.[191] also showed a similarity of different biotypes of fecal Enterobacteriaceae in 51 mother-infant pairs at birth, at one week, and at 2 and 4 months. For the heavily colonized 14 mother-baby pairs we calculated the relative distribution of microorganisms (%) per sample and estimated the predominant ($>10\%$) microorganisms (Fig. 4). We found one or two similar predominant microorganisms in 12 mother-newborn pairs.

Streptococci were the most common predominant microorganisms, occurring in 4 mother-newborn pairs. In no cases did the mothers and their newborns harbor similar potentially pathogenic microorganisms. Lactobacilli, frequently found in mothers, were quite rarely isolated in newborns immediately after birth. The results may depend on the relatively higher resistance of cocci to disinfectants as compared with lactobacilli.[208] Evidently, the idea of decontamination of the birth canal by modern antiseptics (chlorhexidine) before delivery[209] may lead to a selective transfer of maternal bacteria, unfortunately excluding lactobacilli.

Thus, we can assume a close association, both qualitative and quantitative, between the individually different microflora of a mother's perineum and vagina and that of her newborn. Consequently, the predominant pattern of the mother's genital microflora has a significant influence on the initial microecological condition of her newborn.

To date the assumption that the source of the bacteria that finally colonize the infant is the maternal vagina has been revised by the help of discriminatory tests permitting the comparison of bacterial strains isolated from maternal and infant sources. Plasmid profiling has proven a useful technique for this. Tannock et al.[195] compared the plasmid profiles of the microbes of the family Enterobacteriaceal, lactobacilli, and bifidobacteria

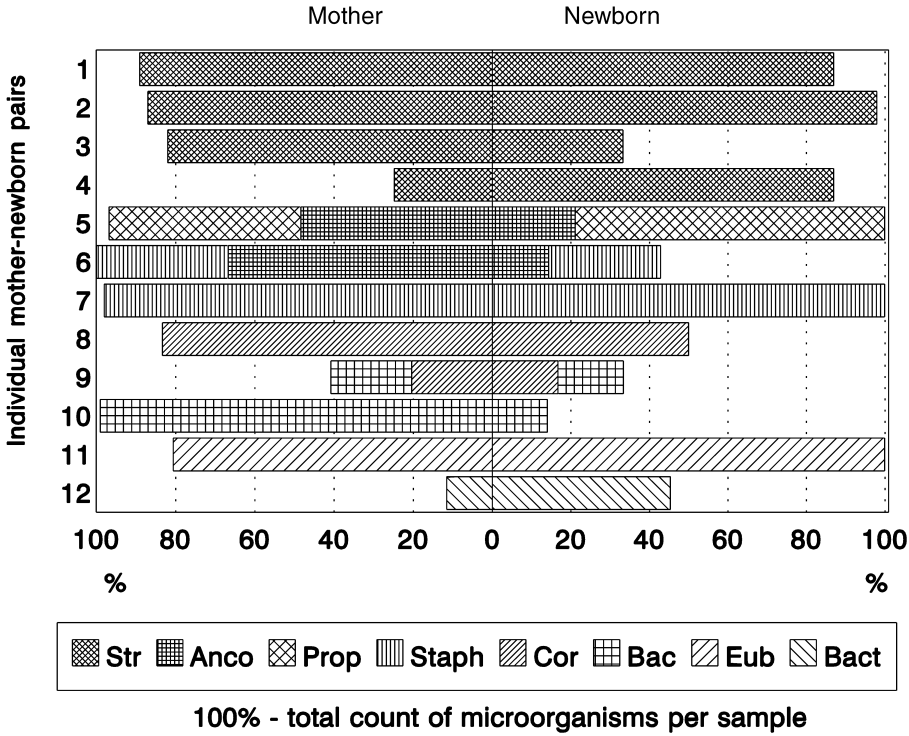

Figure 4 Similar predominant microorganisms in 12 mother-newborn pairs immediately after delivery. Specimens were collected from vagina of mothers and from external ear of newborns.

cultured from the vaginal, oral, and rectal swabs of birth-giving mothers with the strains detected in the feces of their infants 10 and 30 days after birth. Lactobacilli inhabiting the mothers' vaginas did not appear to colonize the infants' digestive tract, but the authors found evidence of the transmission of fecal isolates of enterobacteria and bifidobacteria from mothers to their infants.

Matsumiya et al.[135] performed an interesting study comparing vaginal lactobacilli of pregnant women at the end of pregnancy and their babies' fecal lactobacilli at the age of 5 days and 1 month. Of 86 pregnant women tested, 71 (83%) were vaginal lactobacilli-positive. Five days after birth, 29% of infants had lactobacilli—34% of the babies of lactobacilli-positive mothers and only one of the 15 infants of lactobacilli-negative mothers. After one month, 45% of all children harbored lactobacilli, including 9 children of 15 lactobacilli-negative mothers. Arbitrarily primed (AP) PCR typing revealed that at 5 days after birth only a quarter of infants had similar strains to their mothers and that only 2 of the infants retained the same vaginal lactobacilli until 1 month of age. This study suggests the possible importance of environment or mothers' skin and breast milk as sources of lactobacilli after birth.

2. Cesarean Section and Prematurity

Cesarean section thoroughly alters the colonization patterns in newborn infants. Anaerobic colonization is delayed, and there appears an overgrowth by enterobacteria.[210] The rates of colonization by *Bacteroides fragilis*, *Bifidobacterium*, and *Lactobacillus* are signifi-

cantly lower during the first few months of life in infants born by cesarean section than in vaginally born infants.[211] The colonization of newborns delivered by cesarean section occurs during the first days of life, mainly by bacteria provided by the external environment.[212] While Lennox-King et al.[213] found nurses' hands and contaminated air to be the most common sources for *E. coli* colonization, Bezirtzoglou and Romond[214] doubt the role of hospital environment and feeding type in Enterobacteriaceae colonization. Torres-Alipi et al.[215] found no correlation between the microorganisms isolated from the amniotic fluid and the neonate's oral cavity after a cesarean section.

In premature infants, the acquisition of oral bacteria (mainly CONS and non–*E. coli* gram-negative bacteria) increased from day 1 to day 10 of life, regardless of gestational age and antibacterial therapy.[216] Also in preterm, extremely low-birthweight newborns, the mode of delivery did not affect fecal microflora at day 10 of life.[205] In the case of neonates obtained by vaginal delivery, there was, however, a correlation between the microorganisms of the oral cavity and the maternal vaginal flora. This points to the crucial role of maternal vaginal flora in early contamination of preterm newborns with microbes.

B. Successive Colonization of Infants by Microbes During the First Years of Life

1. Intestinal Tract

To our knowledge no studies have followed the successive colonization of gastric or small intestinal microflora during infancy. All available investigations concern the fecal microflora development, mostly comparing its development in breast-fed infants to that in babies fed with various formulas.[181,190,197,198,217–219]

The frequency of occurrence of lactic acid bacteria in the first months of life is variable according to the data provided by different authors (15–100%) and is higher with formula feeding.[220–222] These data contradict an earlier suggestion that in breast-fed infants bifidobacteria predominate from the end of the first week of life.[181,223–225] Alternatively, the investigations of Simhon et al.[226] and Gothefors[227] showed no difference between fecal microflora of breast- and formula-fed English infants, as in both groups *Bacteroides* were the predominant microorganisms. At the same time, in Nigerian children bifidobacteria were observed to predominate in fecal flora.[226] Similar findings were reported by Adlerberth et al.[42]

Favier et al.[198] demonstrated, on the basis of molecular methods, that though newborns were colonized with *E. coli*, *Enterobacter asburiae*, *Veillonella dispar*, and clostridia on the first day of life, after a few days on a breast milk diet there was subsequent colonization with bifidobacteria. Satokari et al.[206] also demonstrated using molecular methods that the distribution of *Bifidobacterium* and *Lactobacillus* species was similar in breast- and formula-fed infants. The most frequently found representatives of these genera were *B. infantis* and species belonging to the *L. acidophilus* group in both groups of infants. Undoubtedly, the composition of infant formula has been improved over the last 10 years, which could have led to a more breast-fed–like colonization pattern in formula-fed infants.

Investigators of microbial metabolism have also proven the protracted step-by-step formation of the GI microflora of neonates and infants. The microbial short-chain fatty acid (SCFA) composition in the feces of neonates is described as quite specific with a high concentration of acetic acid, connected with aerobic metabolism. As neonates grow older, the composition changes, becomes more complex, and displays wide interin-

dividual variations.[228,229] The mucin-degrading capacity is one of the microflora-associated characteristics (MAC) of the host, which serves as a good indicator for the study of microbial ecology of the intestine.[150,230] Norin et al.[231] followed the mucin-degradation process from birth and found that mucin breakdown starts only at the second year of life. It was shown that in children exclusively breast-fed for at least 4 months, mucin degradation was initiated significantly later than in children who received formula. A positive correlation between increasing age and increased degradation of mucin was found between birth and 1 month and between 6 and 9 months.[229] Thus, the metabolite studies also confirm that the process of microflora formation of infants is individually different.

We studied the predominant ($>10\%$ of the total count in CFU/g) populations of fecal microorganisms from 18 infants at different times over a year.[232] Wide interindividual variations were observed, just as in the biochemical studies of fecal SCFA of the same infants.[153] In several infants we detected the same predominant groups of microbes during the investigation period. Yet lactobacilli were never among the predominant fecal microflora of the infants investigated by us.[233]

2. Geographical Differences in Neonates/Infants Microflora

In a prospective study of neonates in Pakistan and Sweden, it was found that the Pakistani infants were colonized significantly earlier and with more species of enterobacteria than the Swedish babies.[42] This was independent of the mode (vaginal, cesarean section), place (at home, at hospital) of delivery, or diet. In countries with different levels of industrialization, some diverse trends in the numbers of lactobacilli in children have been reported. Early and intense colonization of the intestinal tract by streptococci from the lactic acid bacteria group has been found among Guatemalan Indians,[223] with a higher prevalence of lactobacilli in Ethiopian children as compared to Swedish.[41]

Similarly, we have found remarkable differences in the establishment of lactic acid microflora between Estonian and Swedish children. The differences in colonization by lactic acid microflora could be divided into two phases: neonatal and 12 months. First, at one-week of age substantially higher counts of enterococci and bifidobacteria were found in the feces of Estonian babies than in their Swedish counterparts (Table 3).

In Estonian newborns the counts of coagulase-negative staphylococci and enterobacteria were also higher than in Swedish one-week-old newborns. Moreover, a higher colonization rate with lactobacilli was shown in Estonians at one month of age.[38]

In the first phase of establishment of microflora, the indices of LAB of Estonian neonates resemble those of Guatemalan Indians and Ethiopian children.[41,223] It is likely that the reason may lie in the degree of bacterial exposure during the neonatal period. Lundequist et al.[220] indicate an ecological imbalance of women's vaginal microflora in developed countries and the excessively high hygiene at delivery, which eliminates the normal microflora otherwise acquired from the mother. Adlerberth et al.[42] have suggested that the hygiene conditions at birth and the early breastfeeding are likely to influence the intensity of intestinal colonization in developing versus industrialized countries. However, obstetric practice does not differ between Estonia and Sweden, indicating other confounding factors.

Second, in cross-sectional and prospective studies of healthy infants[37,38,44,234] BR37 at the age of 1–2 years a trend towards higher prevalence (Fig. 5) and variety of species of *Lactobacillus* was detected in Estonian as compared to Swedish infants (Fig. 6). The main variable in the different succession of microbes seems to be diet. We studied during the weaning period the intestinal microflora of children: the counts of

Table 3 First Aerobic Fecal Bacteria in 1-Week-Old Estonian and Swedish Newborns

	Estonian ($n = 17$)	Swedish ($n = 20$)	
	log CFU/g		p-value
Aerobes	11.7	10.3	0.01
CONS	10.6	8.3	0.003
S. aureus	9.1	6.3	0.006
Enterococci	11.0	9.8	0.0002

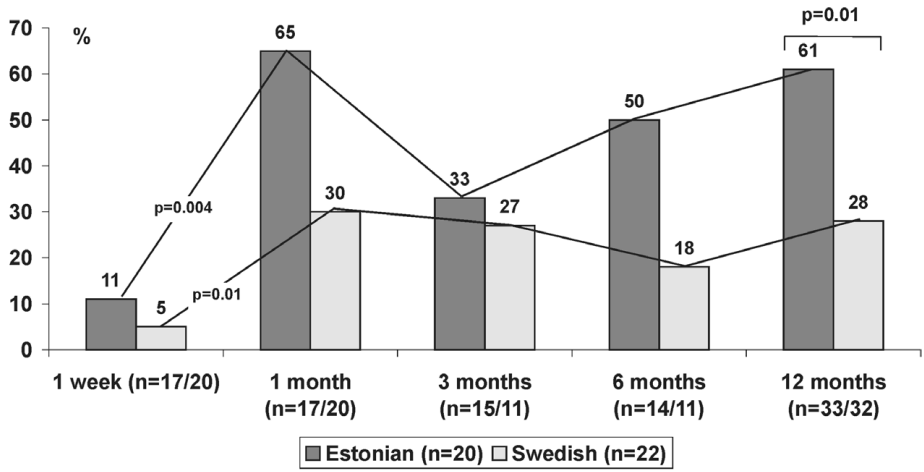

Figure 5 Prevalence (%) of lactobacilli among Estonian and Swedish children.

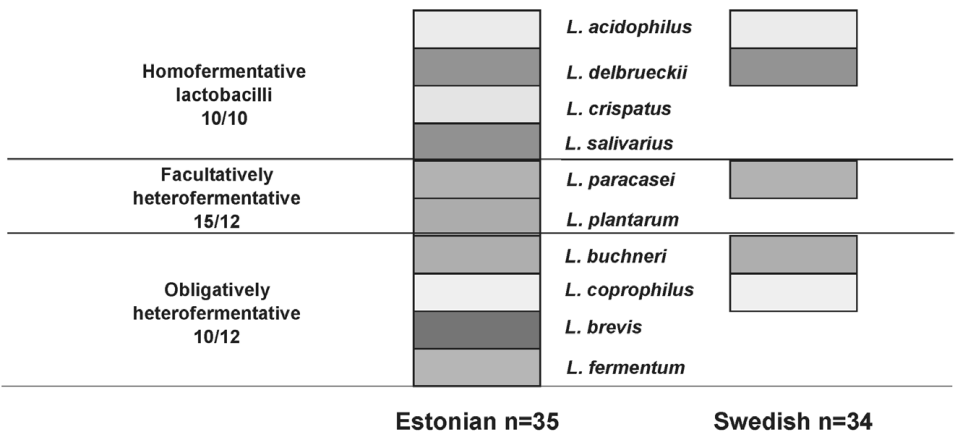

Figure 6 Diversity of *Lactobacillus* spp. in 1- to 2-year-old Estonian and Swedish children.

lactobacilli and eubacteria were higher in Estonian children, and at the same time the counts of clostridia were higher in Swedish 1-year-old children.[37] Yet, the investigated Estonian and Swedish babies did not differ in the frequency or duration of breastfeeding.

When a child starts to eat solid food (mostly from the fifth to sixth month), high numbers of bacteroides, anaerobic cocci, and clostridia are detected in the feces.[235] If breastfeeding is totally stopped, the infants' intestinal microflora succession is broken.[180] In our study of 2-year-old children, the ratio of intestinal anaerobes to aerobes was quite similar to that in adults.[108] According to Lidbeck and Nord,[96] up to the age of 4–8 years, a child's fecal flora resembles that of an adult. However, regional differences may be present. In a comparison of Estonian and Swedish 2-year-old children, the counts of anaerobes, such as bacteroides, bifidobacteria, and clostridia, were higher in Swedish children.[236] According to our studies the intestinal microflora of Swedish children is similar to adults in western industrialized countries, where the count of anaerobes is high and the count of microaerobic bacteria low.[41,166]

The establishment of the more complex mixture of *Lactobacillus* spp. in Estonians than in their Swedish counterparts resembles the data concerning enterobacteria. Once colonized, the Swedish infants often carried a single *E. coli* strain in their microflora for long periods, while Pakistani infants were colonized with a multitude of different *E. coli* strains.[194] In addition to poor hygiene providing the greatest source of bacteria, the genetically determined receptors of cells/mucins may be involved. The increased consumption of locally produced foods and various lactic acid fermentation products starting from the second half of the first year may be an alternative reason for higher prevalence of lactobacilli in 12-month-old infants from Estonia. It is possible that the richer load of lactic acid microflora, both in terms of number and species, in Estonian newborns may have greater importance for the welfare of the child. The wider set of cellular and metabolic antigens of lactobacilli seems to provide protection for the child against harmful substances.

C. Factors Contributing to the Development of Individual Lactoflora

1. Negatively Interfering Factors

Different factors have been shown to influence the development of normal microflora, and one of the factors negatively influencing lactoflora without doubt is antimicrobial treatment. The fact that newborns receiving parenteral gentamicin and ampicillin treatment experience suppression of aerobic and anaerobic fecal flora has been confirmed by Bennet et al.[237] In premature infants treated in intensive care units, the primary colonization of the digestive tract was found to differ from that of infants in puerperal wards,[238] and many frequently used antibiotics have been found to significantly alter the fecal flora, including lactobacilli, of 1- to 3-month-old infants.[239] Alteration of physicochemical surface properties by even sub-inhibitory concentrations of antibiotics may lead to a disruption of the indigenous microflora and creation of a more pathogenic biofilm.[240]

Ahtonen[183] succeeded in showing that ampicillin administered *intra partum* to the mother was shown to delay the newborn's gastrointestinal colonization by *Lactobacillus* species. By using antimicrobial agents that do not disturb colonization resistance, the risk of emergence and spread of resistant strains between patients and dissemination of resistant determinants between microorganisms is reduced.[241]

We have studied the antimicrobial susceptibility pattern of lactobacilli in pregnant women. In some situations, such as urinary or genital tract infections, a pregnant

woman must be treated with antimicrobial drugs, although it is advantageous to avoid the use of antibiotics highly active against lactobacilli. Our results[105] correspond to data from other studies[133,242,243] showing that vaginal lactobacilli are not uniform as to their susceptibility to antibiotics and that it is difficult to predict their sensitivity pattern.[244]

Quite similar data were obtained from studying the antibacterial susceptibility of intestinal lactobacilli in 1- to 2-year-old Swedish and Estonian children.[245] Sixty isolates (10 species) of lactobacilli (29 Estonian and 31 Swedish strains) and 5 collection strains were tested against 9 antibiotics in microaerobic conditions (Table 4). All of the tested lactobacilli were susceptible to ampicillin, gentamicin, and erythromycin and resistant to metronidazole. Single strains were resistant to cefuroxime and tetracycline, about half of the strains to cefoxitin and ciprofloxacin, and three quarters of the strains to vancomycin. We noted that all cefuroxime-resistant strains appeared to be *L. brevis*, but cefoxitin-resistant strains belonged to
a different species. All vancomycin-susceptible strains belonged to the OHOL. Susceptibility of the collection strains did not differ from that of the strains under investigation. Susceptibility for the Estonian and Swedish strains was not significantly different ($p > 0.05$).

Different investigators have found some strains of lactobacilli to be resistant to vancomycin,[243,246–249] which was also revealed by our studies. This property seems to be species-related. Simoes et al.[250] have found that high concentrations of metronidazole (>5000 μg/mL) suppressed the growth of lactobacilli, but concentrations between 128 and 256 μg/mL stimulated their growth.

According to our data, ciprofloxacin, metronidazole, cefoxitin, vancomycin seem to be more suitable for preservation of normal lactoflora than the other regularly used antibiotics. However, this list may be somewhat investigator-dependent, since according to Testore et al.[243] the most appropriate antibiotics are aztreonam, cefixime, kanamycin, pefloxacin, and fusidic acid. Concerning the choice of antibacterial drugs for pregnant women in the case of urinary tract infections, the usual ampicillin and cefotaxime treatment does not seem to be appropriate, as it unavoidably damages the vaginal lactobacilli; treatment with aztreonam and/or quinolones seems to be safer for vaginal lactoflora.

2. Microbial Survival in the Host

Colonization of the biotope by particular strains of lactobacilli requires some important microorganism characteristics: (a) ability to survive in the secretions of the host, (b) resistance against antagonistic activity of other microorganisms of microbiocenosis and ability to repress their growth to some extent, (c) colonization of the mucus layer of the mucosa by degradation of the endogenous nutrients of the host to achieving stable population levels, and (d) adherence to host structures such as epithelial cells in crypts.[62,193,251] All of these properties may simultaneously influence the formation of individually specific lactoflora, which makes investigation into the microbial ecosystem of different organs a highly complicated task.

We have found no studies on the bacteriostatic or bactericidal influence of host secretions of a particular biotope on lactobacilli isolated from the same biotope. There may be some specific characteristics of both variables (secretion and microbes) that determine the survival of a specific strain. The survival of mainly commercial lactobacilli in the human stomach and in transition through the intestine has been investigated both in vivo

Table 4 Susceptibility of Lactobacilli According to Fermentation Type and Species

		Susceptible strains (%)		Susceptibility of 5 more frequently isolated species of lactobacilli			
Antibiotic[a]	Fermentation type	Investigated strains ($n = 60$)	Collection strains ($n = 5$)	Species	R[b] (%)	I (%)	S (%)
Cefuroxime	OHOL	100	100	*L. acidophilus* ($n = 15$)	0	0	100
	FHEL	96	100	*L. paracasei* ssp. *paracasei* ($n = 16$)	0	6	94
				L. plantarum ($n = 7$)	0	0	100
	OHEL	79	100	*L. brevis* ($n = 7$)	43	14	43
				L. buchneri ($n = 6$)	0	0	100
Cefoxitin	OHOL	61	100	*L. acidophilus*	27	20	53
	FHEL	0	0	*L. paracasei* ssp. *paracasei*	100	0	0
				L. plantarum	100	0	0
	OHEL	21	100	*L. brevis*	57	29	14
				L. buchneri	0	50	50
Ciprofloxacin	OHOL	17	0	*L. acidophilus*	87	0	13
	FHEL	70	100	*L. paracasei* ssp. *paracasei*	0	6	94
				L. plantarum	71	14	14
	OHEL	26	0	*L. brevis*	71	29	0
				L. buchneri	17	33	50
Tetracycline	OHOL	93	100	*L. acidophilus*	0	7	93
	FHEL	83	100	*L. paracasei* ssp. *paracasei*	0	0	100
				L. plantarum	29	29	43
	OHEL	63	50	*L. brevis*	14	57	29
				L. buchneri	17	17	67
Vancomycin	OHOL	89	100	*L. acidophilus*	7	0	93
	FHEL	0	0	*L. paracasei* ssp. *paracasei*	100	0	0
				L. plantarum	100	0	0
	OHEL	0	0	*L. brevis*	100	0	0
				L. buchneri	100	0	0

[a]None of the tested lactobacilli was resistant to ampicillin, gentamicin and erythromycin; all strains were resistant to metronidazole.
[b]R, resistant; I, intermediate; S, susceptible.

and in vitro.[70,252–255] The extreme condition (acidity) of the stomach predicted to select for the biotope-specific *Lactobacillus* strains has not been completely validated. The role of the environment of the small and large intestines in influencing the colonization by lactobacilli was recently reviewed by Johansson[65] and Salminen et al.[72]

One host secretion that can influence the composition of microflora is lysozyme.[99] Practically all of the lactobacilli isolated from the normal microflora of humans are resistant to high concentrations of lysozyme (50 mg/mL), yet the strains of lactobacilli detected in the environment are susceptible (15 mg/mL) to lysozyme. Strains of *L. fermentum* isolated from the intestinal tract are resistant to high concentrations of lysozyme, and at the same time they themselves produce lysozyme.[256] However, variable susceptibility was observed among strains of *L. acidophilus*.[257] Consequently, the different susceptibility or resistance to lysozyme may be one of the factors determining the individuality of the lactoflora.

Interactions Between Microbial Groups of Microbiocenosis. There exist specific interactions between various groups of microorganisms performing particular microbiocenosis. Species composition and the number of microorganisms is mostly influenced by bacterial physicochemical activities. In recent years several studies have been performed or data summarized on the role of primary metabolites and anti-microbial substances synthesized by lactobacilli and their antagonistic activity in microbiocenosis.[58,93,258,259]

In a continuous culture model of fecal microflora, it was shown by Bernhardt et al.[260] that the relative proportion of lactobacilli in the total count of microorganisms was not influenced by admitting into the system biologically active strains of other lactobacilli. Thus, even the isolated colonic content appears to be a well-balanced self-regulating system.[261–263]

Many of the metabolites produced by lactobacilli have broad antimicrobial activity against other species[264] in contrast to which bacteriocins inhibit only closely related species of other gram-positive bacteria.[265] Lactobacilli are thought to be highly competitive due to their production of several metabolites including organic acids, H_2O_2, CO_2, diacetyl, acetaldehyde, reuterin, and bacteriocins. A low molecular weight antimicrobial substance of lactobacilli is described in the fecal strain of *L. rhamnosus* GG.[266] It produces a substance having a potent inhibitory effect on a wide range of bacterial species. A very valuable property of the aforementioned substance is that it is not inhibitory against other lactobacilli of the microbiocenosis.

The inhibitory activity of lactobacilli against commensals or pathogens of either gram-positive or gram-negative origin seems to be very variable and strain-specific regarding both pathogens and lactobacilli.[9] We have found that the antimicrobial activity of lactobacilli in vitro using well-standardized methods depends upon their fermentation abilities and the growth conditions, e.g., microaerobic or anaerobic environment.[267] Both heterofermentative lactobacilli have high antimicrobial activity against various target bacteria, whereas the antagonistic effect for the OHOL group was low. Gram-negative bacteria such as *Escherichia coli*, *Shigella sonnei*, and *Salmonella* Typhimurium were more completely suppressed than the gram-positive *Staphylococcus aureus*, *Enterococcus faecalis*, and other lactobacilli (Fig. 7). There was no strain-specific antagonism found for target bacteria when comparing urinary tract infection–causing *E. coli* isolates with intestinal isolates from healthy children.[267]

Several investigators have isolated highly antagonistic lactobacilli and associated this activity with a decrease in pH.[268] It has been proposed that low external pH

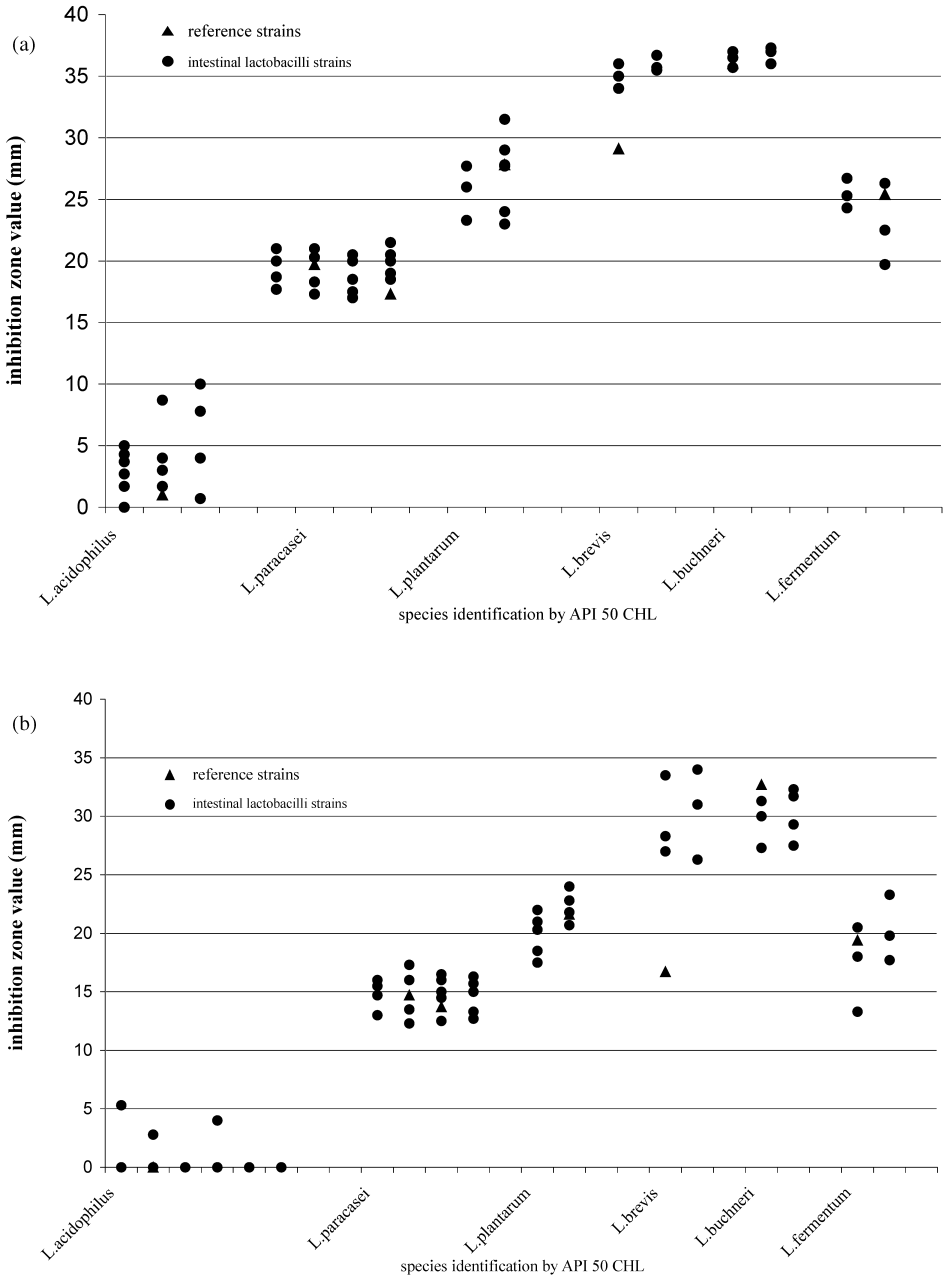

Figure 7 Antagonistic activity of lactobacilli against (a) *Shigella sonnei* and (b) *Enterococcus facealis* on modified MRS agar.

causes acidification of the cell cytoplasm, whereas the undissociated acid, being lipophilic, can diffuse passively across the membrane.[269] The undissociated acid subsequently acts by collapsing the electrochemical proton gradient over the membrane or by altering the cell membrane permeability, which results in disruption of the

substrate transportation systems.[268,270] We found a close correlation between the decrease of pH and antagonistic activity and connected it directly with the production of lactic and acetic acid.[267]

Interactions Between Different GI Microbiocenosis. A high antibacterial activity determined in vitro does not always predict the behavior of a particular strain in the GI microbial ecosystem. Data show that the particular lactoflora of the biotope is created by the interaction of properties of lactobacilli and the ecological niche.[51,62] It has yet to be determined whether the metabolites and bacterocins of lactobacilli are valid in the particular biotope. For example, we have found that representatives of the OHEL group are more active in a microacrobic than an anaerobic environment.[267] Axelsson[271] showed that several strains of *L. brevis* ferment glucose poorly when grown in an anaerobic environment, which may be associated with the low production of antimicrobial metabolites. In contrast, *L. reuteri*, also from the OHEL group, has been found active in an anaerobic environment, which is an important condition for the production of reuterin.[272]

The main variables of the biotope that influence the microbial population level seem to be gas concentration and composition (oxygen, carbon dioxide, methane, hydrogen), pH and Eh (reduction-oxidation potential), and mucosal secretions, such as lysozyme, hormones, immunoglobulins, as well as dietary fiber, urea, and nonmetabolizable sugars.[99,273] In the colon, a zone of physiological stasis, the number of microorganisms is high (10^{10}–10^{12}). The predominant flora is strictly anaerobic (*Bacteroides*, *Eubacterium*, *Fusobacterium*, and bifidobacteria) and the subdominant flora mostly facultatively anaerobic (Enterobacteriaceae, *Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Lactobacillus* spp.). According to Rambaud,[274] the balance between species of microorganisms in the colonic ecosystem and its stability results primarily from microbial interactions.

The administration of *Lactobacillus* GG (10^{10}–10^{11} CFU/g) as a supplement to breast-fed infants during their first 2 weeks of life did not change the relationships between different groups of intestinal bacteria. Although the counts of lactobacilli increased during the administration of *Lactobacillus* GG in comparison with control group neonates, the same could be shown for other intestinal microorganisms, thus keeping their proportion stable.[275] Our finding was confirmed by another, similar study showing the stability of fecal SCFA composition during feeding of premature infants with *Lactobacillus* GG.[276]

It is thought that in different habitats lactobacilli mainly exclude pathogens or potentially pathogenic microorganisms either by occupying the receptors of epithelial cells or by exhausting the nutrients.[277] The colonization of the biotope is mostly influenced by the adhesion phenomenon and, in the case of pathogens, by invasion. Conway[111] suggested competitive colonization, which includes inhibition of growth and colonization. Both aspects seem to be important for the genesis of individually specific lactoflora. The influences of various factors of host and microbial origin participating in the formation of the individual GI microbial ecosystem are interconnected. That creates great methodological limitations for research. However, understanding these different driving forces is important for the selection of suitable probiotic candidates.

Attachment (adherence, adhesion) to solid substrates is a property characteristic of many pathogenic and indigenous microorganisms.[278] By adherence, microorganisms can avoid being swept away by body fluids and may start the invasive process.[279] The presence of lactobacilli in the intestinal, urinary, and genital tracts has been shown to prevent

infection of these systems with specific pathogenic bacteria through a number of mechanisms. Namely, competitive inhibition for bacterial adhesion sites is one of the possible mechanisms of action for lactobacilli against pathogens.[280] The data according to which the less active of two strains with different metabolic activities has to adhere, sometimes evenly, just to the surface of the continuous culture system.[57] seem very relevant. It has also been shown that adherent strains of lactobacilli are likely to survive passage through the GI tract and thus have greater metabolic effects.[281]

Enteric pathogens such as *Helicobacter pylori* and diarrheogenic *E. coli* possess hemagglutinins/lectins on their cell surface, which have specificities for various carbohydrates including sialic acid, galactose, and mannose. We have characterized the cell surface carbohydrates of lactobacilli that could potentially act as receptors for bacterial lectins expressed by pathogens. In our study we confirmed that receptors for Gal or GalNAc are present and available for binding on the surface of *L. fermentum*, *L. paracasei* ssp. *paracasei*, and *L. buchneri*. Furthermore, cell-surface proteins may also obscure carbohydrate receptors differentially expressed by the strains such as Man, GlcNAc, and sialic acid.[282] The latter receptors of lactobacilli may augment the antimicrobial capacity of these strains through co-aggregation with selected pathogens in both the gut and the urogenital tract. Due to the presence of specified receptors, the strain *L. fermentum* ME-3 (DSM 14241) possesses the potential for prevention of urinary tract infections (UTI). Its resistance to fluorokinolones makes it possible to apply this probiotic during treatment of ongoing infection for prevention of recurrent UTI.[283]

In some animal experiments, we have seen that highly antagonistic lactobacilli still could not eliminate the pathogens from the biotope. Thus, in rats mono-associated with *Bacillus subtilis*, the *Lactobacillus* strains with a high bactericidal effect on this bacterium could not exclude the *B. subtilis* from the mucosal micro flora of the ileum.[246] The *L. fermentum* strain ME-3 did not provide resistance against a highly virulent invasive *Salmonella* Typhimurium strain, though it successfully inhibited its growth in vitro.[49] These data indicate that in the formation of a particular microbiocenosis, the decisive role is played both by particular virulence mechanisms of the pathogen or opportunistic pathogen, e.g., rapid evasion of antagonistic actions of lactobacilli by intracellular invasion, and by the ecological niche and its confounding factors.

The idea of either suppressing specific adhesion of putatively pathogenic microorganisms by naturally indigestible carbohydrates or stimulating the growth of lactobacilli by the same substrates has become an object of intensive investigation.[22,69,281,284–286] A new trend, ecoimmunonutrition, is based on the administration of special diet/formula substances together with bacterial supplementation (the formula based on in vitro fermentation of oats and containing large quantities of live *Lactobacillus plantarum* 299). In severely ill patients this new generation of nutrition was shown to induce recovery without antibiotics.[287–289]

Interactions of Microbial Communities in Reproductive Tract. The major factor controlling the microbial types and population levels in the vagina is generally believed to be the hormonal status of woman contributing to the acid environment of the vagina (pH 4–5). This favors the survival and growth of acidophilic microorganisms, mainly lactobacilli.[127] The influence of hormones on the number and species of lactobacilli has been successfully followed in vaginal microflora studies. A high estrogen level of the fetus generates predominance of lactobacilli in the vagina. The converse is also true—a

decrease of estrogen in postmenopausal women causes the disappearance of lactobacilli from their genital tract.[290,291] Estrogen therapy can restore the earlier situation.[123,292] The importance of hormones for colonization by lactobacilli is vividly proved by data demonstrating that progesterone and estradiol were able in vitro to intervene in the adhesion process of different lactobacilli.[293] The study of Miller et al.[294] has shown that hypoestrogenic state is associated with decreased H_2O_2-positive *Lactobacillus* colonization and slight thinning of the vaginal epithelial layer, these changes possibly compromise the vaginal barrier to infection. Studies have confirmed that the receptor activity of vaginal cells and the total count of vaginal lactobacilli, dependent on the menstrual cycle, seems to be important.[295] Whether or not these processes are connected with the individuality of the lactoflora is not yet clear. However, the source of the typically low pH of the human vagina is a matter that has not been finally resolved. The question remains whether the low pH is the cause or effect of vaginal *Lactobacillus* colonization or vice versa.[177]

Vaginal microflora is certainly also influenced by the composition of vaginal secretions, which include contribution from vulvar secretions, Bartholin's and Skene's glands, transudates from the vaginal wall, exfoliated cells, cervical mucus, and endometrial and oviductal fluids. The following substances are commonly present: NaCl, potassium, sulfates, vitamins, metal ions, mucins, proteins, peroxidases, immunoglobulins, enzymes (glucosidase, amylase, antitrypsin), complex carbohydrates, lipids, and fatty acids. Other factors of apparent importance include redox potential, presence of H_2O_2, CO_2, and blood, inflammatory response, anatomical ultrastructural changes, as well as many other factors.[123,127,177,296]

Since the vagina is an open microbiotope, some exogenous factors surely influence the vaginal microecosystem: contraceptive devices, sanitary methods, surgery in the genital tract, antimicrobial or immunosuppressive treatment, extragenital diseases, coitus, blood group, and others.[297,298]

Host Genetic Influences. There is the possibility that certain genes of the host are involved in determination of the particularly different characteristics of microbiocenosis. There are data suggesting that the individuals differ in harboring special genes controlling their resistance toward pathogenic microorganisms.[299] It has been shown that the mother's genotype is an important factor in determining the fetal outcome of murine cytomegalovirus infection.[300] Also, some strains of mice can be classified as resistant or susceptible to mycobacterial growth. A gene has been identified that controls this resistance in mice mapping to a gene on chromosome 1. The group of genes on human chromosome 2q form a similar group to those of mice.[301] However, we have not found any similar data concerning the indigenous microflora.

Van der Merwe et al.[302] has shown that in cases of Crohn's disease, the quantitative composition of normal fecal microflora is genetically determined. Dutch scientists have also shown that the pattern of antibodies directed to fecal bacteria of different morphotypes were unique for each individual, confirming the genetic influences of the host on their indigenous microflora.[303]

Ethnic differences have also been shown to determine different microflora in the genital tract. In the vagina of women from different ethnic groups (Caucasian, African), Kohlmeyer et al.[304] found different prevalent species, e.g., *L. acidophilus* (white 55%, black 42%), *L. brevis* (0%, 26%), *L. casei* (13%, 6%), *L. delbrueckii* (3%, 1%), *L. fermentum* (17%, 16%), *L. plantarum* (0%, 1%), and *L. jensenii* (12%, 8%). All these data

indicate genetic differences of receptors on epithelial cells according to which microbes select their host.

We have observed that the quantitative composition of fecal microflora of adult monozygotic twins has the same degree of similarity as have the paired samples of a single young healthy person.[6] Monozygotic twins reveal the identity of many genetic markers that are important for the selective colonization of the indigenous microflora.[305] Therefore, both the antigenic structure of somatic cells and secretions of microorganisms as well as the immune reaction are determined by the genotype.[306] In these pairs of twins we have also found a high correlation ($p < 0.001$) of the biochemical activity of microflora. That the composition of microflora depends on host genetics was also proved by molecular methods comparing the intestinal microflora of genetically identical monzygotic twins.[139]

The species composition of monozygotic twins' lactoflora was individually different. Yet in 6 of 10 pairs of monozygotic twins, we succeeded in isolating strains with similar biochemical activity of *L. acidophilus*, *L. casei* ssp. *casei* II, and *L. brevis* I. From two pairs we even isolated two similar strains of lactobacilli: combinations of *L. acidophilus* II with *L. brevis* I and *L. plantarum* II with *L. brevis* II.[112,307] Thus, in the GI tract of adult monozygotic twins, identical strains of lactobacilli could persist. This is probably due to the identical specificity of the host receptors for bacterial adhesins. Consequently, the finding of similar microflora and its biochemical activity in one monozygotic twin proves the genetic influence on the individual microflora.

However, we also isolated *Lactobacillus* strains with identical properties from the GI microflora of piglets of litters of three sows.[307] From the piglets of one litter, five to seven species and biotypes of lactobacilli were isolated. In piglets of two litters we managed to spot identical strains at age 1–5 days and 60–120 days. These strains colonized the epithelium of the stomach and jejunum. These experiments prove both the primary importance of first colonization and that of genetic markers on the formation of individual lactoflora.

The specificity of lactoflora of the various parts of the intestine is not explained solely by receptor-adhesin interactions, but also by the presence of suitable endogenous nutrients. In germ-free mice we have observed the same tendency: despite the use of the particular *L. fermentum* and *E. coli* strains originating from the intestine of Balb-c mice, their quantitative relations in various biotopes of the gastrointestinal tract were individually different (unpublished data). So even pheno- and genotypically identical strains develop an individually different structure of microbiocenosis, depending, it seems, on the host's biotope characteristics. At the same time, the individually specific mucin composition is obviously why the different microbiocenoses of particular individuals integrate into one complete microbial system.

IV. IMPACT OF GI MICROBIAL ECOSYSTEM ON HOST

A. Pathogenic Potential of *Lactobacillus* Species

The long history of safe use of many different species of *Lactobacillus* has given them generally-recognized-as-safe (GRAS) status.[308] However, the discovery of lactobacilli in unusual biotopes creates some controversy. Lactobacilli are regarded as harmless microorganisms, but some authors connect their presence in the bloodstream with bacteremia and endocarditis.[309–311] In the majority of cases there is accompanying preexisting

heart valve damage or strong immune deficiency. We have found *Lactobacillus* bacteremia immediately after extraction of tooth roots in chronic periodontitis and consider it transitory, caused by the translocation of microbes after the disruption of the mucosal barrier of the mouth.[312]

The simple fact of isolating lactobacilli from an empyema of the gallbladder,[313] from amniotic fluid,[314] or from a pelvic abscess[375] cannot prove their pathogenicity because some other poorly recognizable agents may have been overlooked. In two large surveys, lactobacilli represented less than 0.2% of all isolates found in blood cultures.[315,316] Subsequent investigation indicated that the increased use of the probiotic *Lactobacillus* GG has not led to an increase in *Lactobacillus rhamnosus* bacteremia in Finland.[317]

The resistance to host innate defense systems, i.e., phagocytosis, is not similar in all species of lactobacilli, as shown by experimental animal studies.[318] This suggests new approaches, particularly assessment of macrophage bactericidal activity against different *Lactobacillus* strains and use of different experimental infection models to conclude the safety of a particular probiotic strain.

The main species of *Lactobacillus* with some pathogenic potential are considered *L. plantarum* [319] and *L. casei*.[313] *L. paracasei* was continuously isolated from peritoneal fluid obtained at ambulatory peritoneal dialysis of an immunocompromised person with recurrent peritonitis.[320] The experiments of Türi et al.[321] showed proliferative tissue reaction, surrounded by macrophages, lymphocytes, and proliferating fibroblasts, to the inoculation of *L. casei* into the testes of guinea pigs. The same type of proliferative reaction was seen when *L. fermentum* were inoculated into guinea pig testes together or before *E. coli.* However, *E. coli* caused multifocal necrosis of germinative tubules, surrounded by multiple of neutrophiles and eosinophiles if injected as pure culture.[322] Though *L. fermentum* did not reduce the local inflammation towards *E. coli*, it changed the type of inflammation and speeded up the clearance of *E. coli* from blood and tissue. Thus, *Lactobacillus* spp. can cause inflammatory changes of proliferative type after translocation into extraintestinal sites and stimulate the systemic clearance of pathogens, putatively through some immunological mediators. Showing that *L. plantarum* (strain 299v) had no role in endocarditis animal model, the safety of this probiotic strain was assessed.[323] However, for safety reasons every new probiotic strain of *Lactobacillus* should be controlled in different animal experiments.

Dental caries is a widespread disease related to lactobacilli. The complexity of the bacterial community in dental plaque of humans has made it difficult to determine the single bacterial agent of caries, but there is considerable evidence that mutans streptococci are involved in the initiation and lactobacilli in the progression of caries. They are able to rapidly metabolize carbohydrates into acid and to tolerate a low-pH environment.[100] The demineralization of teeth is caused by organic acid produced from the bacterial fermentation of dietary carbohydrates. Lactobacilli are not homogeneous in terms of their cariogenic potential, as certain species, such as *L. casei*, *L. fermentum* and *L. rhamnosus*,[116] have been more frequently associated with caries lesions. Some lactobacilli strains have been found to be antagonistic against mutans streptococci, and they may even reduce the caries risk.[324] Ahumada et al.[325] have shown that lactobacilli from caries-active subjects showed greater potential to adhere and lower production of inhibitory substances, while lactobacilli from caries-free subjects were able to inhibit potentially pathogenic microorganisms. Therefore, the presence of lactobacilli in saliva may not be directly related to caries risk if their species composition and properties are unknown.

Though oral lactobacilli have been associated with dental caries, their role in periodontal diseases has been poorly described. In our recent study, half of chronic periodontal disease (CPD) patients but 70% of healthy persons harbored high counts or very high counts of lactobacilli in saliva.[119] The isolated 106 strains belonged to 9 species (Fig. 8). The species range was significantly wider in healthy persons than in diseased ones (median 2.7, range 1–5 vs. median 1.9, range 1–3, respectively). The OHOL strains were more frequent in healthy subjects (70 vs. 17%; $p = 0.017$), with *L. gasseri* being the most prevalent (60 vs. 8%, $p = 0.015$). This figure shows the reduced distribution of lactobacilli in periodontal disease, indicating a protective role for indigenous salivary lactobacilli.

B. Health-Promoting Influences of GI Lactoflora on Host

1. Prevention of Infections

The lactoflora has been shown to play an important role in the health of humans.[21] Mainly, the lactoflora is involved in the prevention of various GI infections by forming a colonization barrier[185] against the establishment of microbes with pathogenic potential.[4,326,327] In several clinical trials the concept of microbial interference therapy (MIT) has been successfully applied. The affects include either maintenance of health or restoration of diseased functions of host by introducing living microbes known as probiotics and having some therapeutic potential. Lactic acid bacteria (*Lactobacillus*, *Enterococcus*, *Bifidobacterium*, *Streptococcus* spp.) and some yeasts (*Saccharomyces boulardii*) are used in probiotic preparations either singly or in combination.

MIT has been applied in many infectious diseases, including travelers' diarrhea, antibiotic-associated colitis, *Clostridium difficile*–related infections, rotavirus enteritis, urinary tract infections, vaginosis, among others (reviewed by Refs.[57,328,329]).

The clinical usefulness of *Lactobacillus* GG as adjunct therapy for shigellosis in children has been shown.[330] One of the intestinal infections for which treatment by lactobacilli has been widely studied is antibiotic-associated diarrhea (AAD). *Clostridium difficile* is the main agent of AAD causing colitis and diarrhea in patients extensively treated

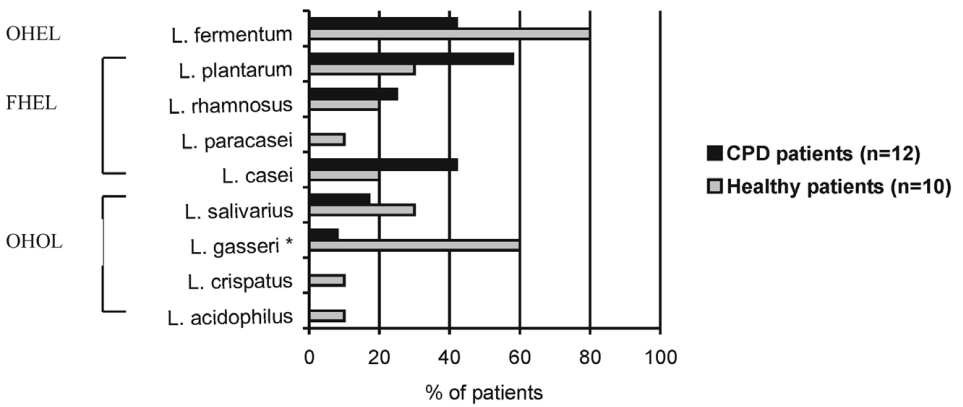

Figure 8 Prevalence of salivary lactobacilli in chronic periodontitis and healthy patients, $^{*}p = 0.015$.

with the antibiotics.[21] Usually the reduction of indigenous anaerobes has been associated with development of AAD.[331,332] In our laboratory it has been shown that the amount of intestinal lactobacilli also seems important in the prevention of *C. difficile* colonization.[333,334] Recently we showed that certain *C. difficile* strains with high vs. low toxigenicity were differentially inhibited by particular *Lactobacillus* strains of *L. plantarum* and *L. paracasei*.[335] It can be speculated that low-virulence strains of *C. difficile* may successfully coexist with some species/strains of lactobacilli in the intestinal tract of some people. However, the highly virulent strains of *C. difficile* could be outcompeted by more effective *Lactobacillus* spp. strains. The AAD seemingly develops if a person does not harbor the highly antagonistic lactobacilli.

The efficacy of probiotic therapy varies largely in different clinical trials. For instance, a well-designed study[336] described a significant decrease in AAD by *Lactobacillus* GG in children, yet similar results were not obtained in adult patients.[332] Reviewing 46 double-blind, randomized, prospective clinical studies with probiotics, a successful clinical outcome was found in only 56% of trials.[337] Besides probiotic efficacy, the proper dose and viability of bacteria and the individually different microbial ecosystems may be one of the main reasons for MIT failure.

2. Role in Noninfectious Chronic Diseases

It has not been determined in clinical studies whether the absence of lactobacilli in fecal samples or their high quantities on the mucosa of the small intestine should be considered an abnormality. Controversial data have been obtained in different clinical situations. Lactobacilli are described as sensitive to emotional stress before and during space flights, their number becoming reduced.[3,338] However, radiation injuries caused by the Chernobyl atomic power plant accident showed, surprisingly, an increase in the counts of intestinal enterococci and lactobacilli, particularly *L. casei* and *L. plantarum*.[339] A similar tendency (i.e., high levels of fecal lactobacilli) was seen in infants with rotavirus diarrhea. The denudation of small intestinal mucosa and subsequent release of mucosal lactobacilli seem to account for the increased numbers of fecal lactobacilli in the situations described. Even *Lactobacillus* GG administration did not increase the total count of lactobacilli.[340]

Noninfectious Gastrointestinal Diseases. Interesting results were obtained by studying the *Lactobacillus* content in patients suffering from some noninfectious GI diseases. Voronina[341] reported that the occurrence of lactobacilli in gastric contents increased in patients with chronic gastritis and gastric cancer, associated with gastric hypoacidity. However, it was found that lactobacilli rarely persist in normal gastric mucosa and also occur rarely in association with *H. pylori* in the heavily colonized mucosa of the antrum.[342,343] Our latest data confirm that lactobacilli are found very rarely in the biopsy material of the gastric mucosa of *H. pylori*–negative adults.[103] The composition of bacterial communities growing on epithelium and mucus of the intestinal tract (mucosal flora) is seemingly determined by a variety of host factors, including cellular and humoral immunity, together with antimicrobial peptides as defensins.[344] that do not have such profound influence, on the luminal flora.

It is likely that the colonization by transient nonindigenous food-deprived lactobacilli may cause, in cases of gastric anacidity the overgrowth-syndrome like situations in the upper part of the intestine. Our experience[143] is consistent with this: increased quantities of fecal lactobacilli were found in cases of anacidity (Table 5). At the same time, in

Table 5 Lactobacilli in the Feces of Patients with Some Noninfectious Gastrointestinal Diseases

	Group of persons	*n*	Frequency of occurrence (%)	Number (log CFU/g)		
				Range	Median	Mean
I	Young healthy persons, 21–44 y	10	98	5.5–8.1	6.6	6.7 ± 0.9
II	Old healthy persons, 65–89 y	13	100	6.0–8.0	7.0	7.2 ± 0.9
III	Chronic gastritis, anacidity, 21–69 y	10	100	7.0–9.0	8.0	8.3 ± 0.7[a]
IV	Carcinoma ventriculi, 50–74 y					
	Anacidity	8	100	7.8–10.0[b]	8.8	8.8 ± 0.9[a]
	Hyperacidity	6	100	5.7–8.0	7.3	7.1 ± 0.8
V	Peptic ulcer, 22–68 y					
	Normacidity	3	100	6.1–7.0	7.0	6.8 ± 0.5
	Anacidity	5	100	7.1–9.8[b]	7.8	7.7 ± 1.2
	Hyperacidity	14	100	5.0–8.0[b]	5.8	6.6 ± 1.1
VI	*Status post–resectionem ventriculi*					
	Without complaints, 35–62 y					
	Anacidity	6	100	8.7–10.0[b]	9.6	9.5 ± 0.6[a]
	Hyperacidity	4	100	7.7–8.7	8.3	8.2 ± 0.4
	With complaints (dumping syndrome), 24–64 y					
	Anacidity	23	100	5.8–10.6	7.5	7.6 ± 0.9

[a] $p < 0.01$ Student's t-test
[b] $p < 0.05$ Mann-Whitney test.
Source: Mikelsaar, 1969.

hypoacidic patients with a dumping syndrome after gastric surgery, the number of lactobacilli was not increased, seemingly due to the intensive clearance of the intestine. Yet these studies are regrettably limited by the lack of real understanding of the function of lactoflora of a particular biotope.

3. Antiallergic Effects

Indigenous microflora, including lactobacilli can nonselectively facilitate natural immune responses. Finnish investigators have shown that oral intake of lactobacillar supplements reverses increased intestinal permeability and by this mechanism could stimulate local immune responses.[345,346] Some control of the adverse effects of radiation has been shown by substitution of lactobacilli preparations.[347] These supplements obviously affect the microbial ecosystem of the GI system of a particular host, correcting either the developed imbalance of microflora or modulating the immune responses. This aspect has been applied to the prevention and treatment of allergy since the early 1990s, based mainly on empirical experience with probiotic use. Several clinical trials of probiotic administration have been conducted.[348,349] The majority can be divided in two groups: preventive and treatment trials.

Cross-sectional and prospective studies have shown pronounced differences in the establishment of intestinal microbes in allergic as compared to nonallergic

children.[34,35,43,44] Differences in fecal microflora of patients with atopic dermatitis were also found.[350]

Several groups of intestinal microbes have been identified during our intestinal microflora studies. In children later developing allergy, a low prevalence of lactic acid–producing bacteria such as enterococci and bifidobacteria was assessed during the neonatal period. During infancy the low prevalence of bifidobacteria was accompanied by increased colonization by potentially pathogenic bacteria, such as *Staphylococcus aureus* and clostridia.[44] By the age of 2 years a decreased proportion of *Bacteroides* was seen in allergic as compared to healthy children.[43] Our recent study confirms the previous findings, showing in 5-year-old allergic children the low prevalence and numbers of bifidobacteria to be accompanied by an increased proportion of clostridia in the gut microflora.

We suggest that at different periods of life (neonate, infant, 1- to 5-year-olds) particular intestinal microbes are involved in (a) the induction of immunological tolerance to intestinal contents, (b) allergic sensitization, and (c) the manifestation or downregulation of mast cell reactions to allergens. During these phases of allergy development, correction by probiotics of microflora shifts could help prevent atopic responses and allergic disease.

In our prospective study,[44] like that of Finnish researchers,[35] a low prevalence of lactic acid–producing bacteria such as enterococci and bifidobacteria was assessed during the neonatal period in children later developing allergy. During the neonatal period, the high numbers of the first intestinal colonizers may support the development of oral tolerance, understood as the exclusion of some T-cell clones or induction of their hyporesponsiveness towards these gram-positive intestinal microbes, their metabolites, and similar luminal soluble antigens.[351,352] Due to reduced microbial pressure, oral tolerance might not be induced and as a result faulty programming of the mucosal immune system could be related to the later developed allergy. The crucial role of microflora in tolerance induction has been shown by the lack of oral tolerance in germ-free animals. Oral tolerance was achieved when bifidobacteria were inoculated into these mice at the neonatal stage, but not at an older age.[352a] A trial[353,354] in which antenatal probiotic administration to pregnant women succeeded preventing allergic disease could be explained by correction of this phenomenon. However, atopic eczema was not fully prevented by probiotic treatment but simply cut in half in pregnant women/infants given *Lactobacillus* GG,[35] hinting at several undiscovered mechanisms.

The low prevalence of bifidobacteria accompanied by increased colonization with potentially pathogenic bacteria such as *S. aureus* and clostridia in allergic children can be seen as important factor for allergic sensitization due to the bacterial components and superantigens, which are able to translocate and modulate eukaryotic cell cytokine synthesis and immune response. At the phase of downregulation of allergic sensitization, the relevant operating mechanism for bifidobacterial probiotic administration may be either the competitive exclusion of potentially pathogenic bacteria or modulation of the immune response.[36] Inhibition of the systemic translocation of potentially pathogenic bacteria by probiotic *Lactobacillus* GG administration[355] may also participate in downregulation of mast cell reactions.

In adults, the probiotics can downregulate the manifestation of atopic reactions due to certain properties of probiotic strains. We have demonstrated the downregulation of atopic reactions by administration of the antioxidant *L. fermentum* ME-3 (DSM 14241) strain to patients with atopic dermatitis.[356,357] However, the effect may depend largely on the

species, the type of introduced probiotic strain(s), the cell wall structure, and metabolites excreted.

4. Antioxidative Effects

An antioxidative effect of lactobacilli has been reported only recently.[48,358–360] A wide variety of reactive oxygen and nitrogen species (ROS, RNS) are continuously produced in the human body, playing a substantial role in the pathogenesis of cancer, cardiovascular diseases, allergies, and atherosclerosis.[361] Virtually all biological macromolecules can be damaged by oxidants. Antioxidants are naturally occurring or synthetic substances that either inhibit or retard the oxidation of other molecules, preventing the formation of radicals by synthesis of reducing species, scavenge the reactive species, or promote their decomposition.[362,363] The well-known antioxidants are compounds with phenolic structures (tocopherols, flavonoids, phenolic acids), nitrogen compounds (alkaloids, chlorophyl derivatives, amino acids, and amines) or carotenoids as well as ascorbic acid.[364] Radical scavenging antioxidants have an advantage when compared to preventive antioxidants (suppression of the formation of free radicals) and the repairing antioxidant enzymes.

Lactobacillus fermentum strain ME-3 (DSM 14241) possesses substantial antimicrobial and antioxidative activity, expressing manganese superoxide dismutase, eliminating hydroxyl radicals, and containing reduced glutathione, a potent cellular antioxidant.[48] Thus, the antioxidative potential of strain ME-3 is generated by at least two different functions (radical scavenging and a glutathione system).

We have demonstrated the downregulation of atopic reactions by administration of the antioxidative *L. fermentum* ME-3 (DSM 14241) strain to patients with atopic dermatitis. In these patients the increased levels of IgE and allergen complexes bind to mast cells and induce the release of histamine and other inflammatory mediators. Inflammation is closely associated with phagocytosis and oxidative burst and creation of ROS. Oral administration of antioxidative lactobacilli[356] was beneficial at sites of allergic inflammation in alleviating tissue damage caused by host-derived excess of oxidants. Simultaneously, blood antioxidative indices (total antioxidative status and reduced glutathione level) were significantly increased in sera and significant reduction of iron was seen in skin of patients after probiotic therapy.[356] Iron as a potent pro-oxidant released from acute phase iron-storage and transporting proteins (ferritin) and transferrin is involved in the rapid formation of ROS.[365]

Free radicals are closely associated with lipid peroxidation. Free radicals such as superoxide anion and hydroxyl radical directly decrease reduced cellular glutathione (GSH) levels and increase the lipid peroxidation in blood sera. Oxidized low-density lipoproteins (ox-LDL) play a crucial role in the pathogenesis of atherosclerosis, being highly atherogenic. This directly damages the endothelial cells, facilitating the conversion of macrophages to foam cells and eventually to fatty streaks.[366]

Lin and Chang[359] reported the inhibitory effect of *B. bifidum* ATCC 15708 and *L. acidophilus* ATCC 4356 on plasma lipid peroxidation. We showed in a volunteer trial that consumption of goat milk fermented by *L. fermentum* ME-3 (DSM 14241) prolonged the resistance of lipoprotein fraction to oxidation, lowered levels of peroxidized lipoproteins, oxidized LDL and glutathione-redox ratio, and enhanced total antioxidative activity. It lowered the level of 8-isoprostanes in urine. During 3 weeks of consumption the persistence of *L. fermentum* strain ME-3 in the intestinal tract was assessed in all volunteers. This caused an interaction between the cells of host and the ME-3 or its metabolites

expressing antiatherogenic response.[50] In an experimental mouse model of *Salmonella* Typhimurium infection, we were able to balance the antioxidativity of damaged gut mucosa during the oral administration of *L. fermentum* ME-3.[49] This could explain how strain ME-3 can exert both antioxidative and antiatherogenic effects.

Recently we performed another randomized, double-blind, placebo-controlled volunteer trial with ME-3 as an encapsulated food additive. Oral consumption three times daily of 10^9 viable cells for 10 days resulted in a significant reduction in 8-isoprostane levels in urine.[367] Isoprostanes are known as good indices of body total oxidative-stress–based atherogenicity.[368] These promising results indicate that several functions of indigenous microflora are yet to be described. More systematic investigations are necessary to establish the molecular mechanisms of probiotics.

C. Influence of Genital Tract Lactoflora on Host

A widespread pathological condition closely related to lactobacilli is bacterial vaginosis (BV). Despite numerous studies, its nature remains unclear, and it has been called an "ecologic mystery".[369] BV is an imbalance of the vaginal microecosystem in which microaerophilic lactobacilli are absent, while there exists a predominance of *Gardnerella vaginalis*, *Mobiluncus* spp. *Mycoplasma hominis*, and some anaerobic microorganisms.[126,177,297] It is not clear which is of primary importance—the appearance of infectious microorganisms or the disappearance of lactobacilli. It has been suggested that some aerobic microorganisms can competitively suppress lactobacilli, enabling further overgrowth of BV-associated microorganisms.[131] Studies have revealed that lactobacilli from patients with BV are unable to cleave glycogen with the production of lactic acid[370] or to produce hydrogen peroxide.[296] Some changes in the host status that lead to decreased availability of immune functions could also trigger the shift in flora.[296] Bleeding, douching, and intercourse may increase vaginal pH and decrease colonization by lactobacilli. In particular, low pH (4.0 vs. 7.2) significantly increases the binding of lactobacilli to fibronectin, which is responsible for lactobacillar colonization of the vagina.[371,372]

We have investigated simultaneously the incidence of BV and the quantitative composition of vaginal microflora in 42 women repeatedly (4–7 times) during pregnancy.[134] Bacterial vaginosis was found in 31.3% of the 229 samples, which corresponds to the data provided by other authors who found BV in 10–26% of pregnant women.[373] At least one episode of BV during pregnancy occurred in nearly half of 42 women, 7 of whom had BV in all samples. The incidence of BV decreased during pregnancy, while the incidence and number of lactobacilli in the vaginal microflora increased ($p = 0.01$). In many women the BV was unstable, showing that its treatment during pregnancy could be postponed until the diagnosis of BV was confirmed repeatedly.

Concerning the idea of "ecological" treatment of BV by administration of lactobacilli into the vagina, studies have shown conflicting results. Commercial probiotic lactobacilli, usually originating from the gastrointestinal tract, frequently fail to colonize the vagina. However, lactobacilli from the urogenital tract have shown quite good effect, even when administered orally.[374]

V. SUMMARY

Humans harbor an individually specific lactic acid microflora in various organ systems, especially in the gastrointestinal and genital tracts. This lactoflora consists of several

species combinations typical for each individual, and its quantity is relatively stable during long survey periods. Unfortunately, the direct investigation of the lactoflora of various biotopes, e.g., GI, is often complicated.

The formation of individually specific lactoflora starts in the perinatal period due to the selective microbial colonization of the baby with its mother's lactobacilli. The selective colonization of the biotope is a characteristic of both the host and the microorganism. This conclusion is based, first, on the demonstration of the importance of the genetic background of the host and, second, on the specific microbial properties, including an ability to survive in the host's secretions and to withstand the antagonistic activity of other microorganisms, involved in the process of individual lactoflora formation. However, more detailed attention to the precise mechanisms connected with the transfer of indigenous microflora from the mother to its neonate is needed to clarify the role of specificity in lactoflora formation.

The precise purpose of the above studies is to learn how to create by means of particular lactobacilli the high colonization resistance of neonates. The ecological imbalance of the mother's microflora, often caused by wide-spectrum antibacterial treatment, on the one hand, and the use of big maternity clinics with their strict antiseptic procedures at delivery, on the other, have distorted the natural source of the normal microflora. To overcome these problems, early close contact of the neonate with its mother immediately after birth has been suggested.

The studies of development of lactoflora are closely related to the question of the impact of lactobacilli on the well-being of the host. Their important role has been demonstrated in communities with different degrees of industrialization. Particularly, the prospective studies of infant colonization by indigenous microflora in children later developing or not developing allergy show clear differences. The possibility of preventing allergy or treating it by probiotics has attracted wide attention by researchers and practitioners.

The discovery of antioxidative lactobacilli widens our possibility of influencing the health of large populations by introducing functional foods with particular claims. The wide area of normal microflora of host may hold many promising secrets and possibilities for new discoveries.

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Lactic Acid Bacteria and Intestinal Drug and Cholesterol Metabolism

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I. INTRODUCTION

The intestinal microflora of humans is a complex ecosystem of metabolically active microorganisms in close proximity to an absorptive mucosal surface. Substrates for bacterial transformation can reach the intestinal flora through direct oral ingestion, by biliary secretion into the upper bowel, or by secretion across the mucosa. This chapter reviews the metabolic activity of the microorganisms that reside in the intestine and reviews the current knowledge regarding the role of *Lactobacillus* in this environment.

II. BACTERIAL COMPOSITION OF THE GASTROINTESTINAL TRACT

The gastrointestinal tract is inhabited by a diverse bacterial population that constitute a complex ecosystem. More than 400 different bacterial species have been isolated and identified in feces.[1,2] Strict anaerobic bacteria are the most common organisms in the intestinal tract outnumbering facultative bacteria by a factor of 10^2–10^4. In Table 1 the most prevalent microorganisms found at various locations in the human gastrointestinal tract are shown. In healthy individuals the stomach and upper small intestine have relatively low numbers of microorganisms.

Table 1 Distribution and Composition of the Intestinal Flora

Site	Composition[a]	Total number of organisms per mL contents
Stomach	*Streptococcus* *Lactobacillus*	10^1–10^2
Duodenum and jejunum	Similar to stomach	10^2–10^4
Ileal-cecal	*Bacteriodes* *Clostridium* Streptococci Lactobacilli	10^6–10^8
Colon	*Bacteriodes* (10^{10}–10^{11}) *Clostridium* (10^{10}) *Eubacterium* (10^{10}) *Peptococcus* (10^{10}) *Bifidobacterium* (10^9–10^{10}) *Streptococcus* (10^{10}) *Fusobacterium* (10^9–10^{10})	$10^{11.5}$–10^{12}

[a]Organisms listed represent only the major species isolated from the different sites.
Source: Ouwehand et al.[37]

The lower small intestine is a transition zone between the sparely populated upper gastrointestinal tract and the heavily bacterially populated colon. In the lower ileum the number of bacteria increases to between 10^6 and 10^7 organisms per milliliter of contents.

In the colon the bacterial concentration increases between 10^{11} and 10^{12} organisms per milliliter of fecal material (Table 1). To illustrate the density of bacteria in the colon, one third of the fecal dry weight consists of variable bacteria.

III. BACTERIAL COLONIZATION OF THE GASTROINTESTINAL TRACT

Colonization of the gastrointestinal tract in humans occurs within a few days after birth (Haenel, 1975). The course of colonization is influenced by gestational age, type of delivery, and dietary constituents. The initial phase of colonization occurs over approximately a 2-week period. During this period the bacterial colonization is similar for breast- and formula-fed infants. Almost always *Escherichia coli* and *Streptococcus* are the first organisms detected in the feces at concentrations between 10^8 and 10^{10} organisms per gram of feces.[3] This is often followed by the appearance of anaerobic organism, namely *Clostridium*, *Bifidobacterium*, and *Bacteriodes*. In breast-fed infants there follows a period during which there is a significant reduction in the populations of *E. coli* and *Streptococcus* and a partial or complete disappearance of *Clostridium* and *Bacteroides*. This decrease in bacterial populations results in the predominance of *Bifidobacterium* in the intestine of breast-fed infants. In formula-fed infants the bacterial reductions and disappearances do not occur, resulting in a more complex intestinal microflora.[4–8] The relatively simple flora of the breast-fed baby continues until other foods are included in the diet. After the introduction of other foods there is a return of *E. coli*, *Clostridium*, and *Streptococcus* to

the intestinal tract of the breast-fed infant as witnessed by isolation of these organisms from the feces.[9] The intestinal flora of the breast-fed infant now resembles that of the formula-fed baby. There is then a period of transition which continues into the second year of life at which time the composition of the intestinal microflora evolve to resemble the bacterial composition found in the adult.

IV. LACTOBACILLI RESIDING IN THE HUMAN GASTROINTESTINAL TRACT

The indigenous intestinal microflora of most healthy individuals harbor representatives of *Lactobacillus* genera. Finegold et al.[1] reported finding in feces *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. minutus*, and other *Lactobacillus* species (not identified). The concentration of viable organisms varied between 10^8 and 10^9. Holderman and Moore[2] also analyzed fecal specimens and found similar results. They also detected *L. leichmannii* and *L. ragosae* in the fecal flora. It is interesting to note that *L. bulgaricus*, the organism commonly used in the production of yogurt, was not routinely isolated from the fecal cultures. It is clear that *Lactobacillus* is a component of the normal intestinal flora, but only certain species are normally present in the intestinal tract.

V. INTESTINAL REACTIONS IN WHICH *LACTOBACILLUS* HAS BEEN IDENTIFIED AS A PARTICIPANT

Scheline[10] tested a number of different substrates and intestinal microorganisms to determine the type of reactions that specific *Lactobacillus* species catalyze. These investigators found that a *Lactobacillus* species could reduce the double bond in hydroxycinnamic acid, reduce the nitro group of 4-nitrobenzoic acid, and reduce the azo bonds found in methyl red and acid yellow. Pradham and Majumdar[11] reported that *Lactobacillus acidophilus* cleaves the azo bond of sulfasalazine, also known as azulfidine, a drug used to treat patients with ulcerative colitis. These investigators also found that *L. acidophilus* degraded 17.6% of the antimicrobial agent phthalylsulfathiazole and 8% of the antibiotic chloramphenicol palmitate. These investigators also confirmed the previous study and demonstrated that *L. acidophilus* could rapidly hydrolyze the azo bond of tartrazine and methyl red. *Lactobacillus helveticus* and *Lactobacillus salivarius* had similar but lower activity when compared to *L. acidophilus*. The most rapid reaction performed by *Lactobacillus* was the reductive hydrolysis of the azo bond followed by hydrolysis of the amide bond. The least active of the reactions studied was the hydrolysis of the ester bond of chloramphenicol palmitate. Gilliland and Speck[12] studied the ability of *L.* and *Lactobacillus casei* to hydrolyze conjugates of bile acids. They found that all six strains of *L. acidophilus* deconjugated taurocholate, but only one of six deconjugated glycocholate. None of 13 strains of *L. casei* hydrolyzed glycocholate. Lundeen and Savage[13] reported that lactobacilli were responsible for 86% of the hydrolysis of bile acids in the ileum and about 74% in the cecum of mice. Another reaction that *Lactobacillus* has been shown to carry out is dehydroxylation.[14] The dehydroxylation product *meta*-hydroxy phenylpropionic acid was isolated in the urine of gnotobiotic rats fed caffeic acid who had been coinfected with two strains of lactobacilli—a strain of bacteriocides and that group N streptococci. The combination of organisms was required for the dehydroxylation, since none of the individual bacteria carried out the reaction alone. Lactobacilli have also been shown to reduce the double bond of 3-hydroxycinnamic

acid[10] and cinnamic acid[15] to produce, respectively, 3-hydroxyphenylpropionic acid and phenylpropionic acid. *Lactobacillus* species have been shown to be capable of decarboxylating amino acids,[16] and a *Lactobacillus acidophilus* isolate from the stomach of rats was shown to exhibit histidine decarboxylase activity.[17] The biological function of biogenic amines is currently under debate. Although at high concentrations they are detrimental, the body requires them in low concentrations.

VI. INTESTINAL CHOLESTEROL METABOLISM

The intestine has a profound effect on cholesterol metabolism, many areas of which have been reviewed.[18,19] The liver and intestine are the sites of cholesterol synthesis and metabolism. A unique aspect of the intestine is the presence of a large and diverse bacterial population, which impacts greatly on this process. Complicating the understanding of those processes is the extreme variation in the normal intestinal processing of cholesterol by different population groups and among individuals with diseases of the intestinal tract. Additionally, there is variability with respect to the effect of dietary supplements and probiotics on plasma cholesterol concentrations. For these reasons, an understanding of the complex fate of cholesterol in the intestinal tissue and lumen of the intestine is required to understand sterol balance in the body.

VII. SOURCES OF INTESTINAL CHOLESTEROL

A major source of intestinal cholesterol derives from the de novo synthesis of this sterol. In many animal species mucosal cells secrete cholesterol directly into the lumen;[19] however, this does not appear to occur in humans.[20] In humans cholesterol synthesized by the intestinal cells is introduced into the small intestine via exfoliation of intestinal cells. Additional sources of intestinal cholesterol come from secreted bile and the diet. Connor et al.[21] suggested that bacterial metabolism of cholesterol converters was highest in persons consuming a mixed western diet. Subsequent work has indicated that there is not a homogeneous group of converters, and that the percent conversion may be lower than first thought.

VIII. ASSOCIATION WITH BACTERIAL CHOLESTEROL AND COLONIC CANCER

Considerable interest has focused on determining whether bacterial metabolism of cholesterol influences the development of certain disease states of the colon, especially in light of the findings that there exist certain individuals who have markedly different rates of bacterial metabolism of cholesterol. Aries et al.[22] suggested that the composition of the intestinal microflora was dependent on diet and that variations in dietary intakes influenced intestinal secretions and the substrates available to the bacteria for metabolism. Surveys of different population groups with different risks of colon cancer support this hypothesis.[23–25] Reddy et al.[24] found that switching a person from a high-colon-cancer-risk (high meat) diet to a low-colon-cancer-risk (non-meat) diet resulted in shifts in the composition of the intestinal microflora and neutral sterols.

Reddy et al.[24] reported that patients with diagnosed colon cancer who were consuming what was considered to be a mixed western diet had higher total sterol output than controls. This increased excretion of sterols was contributed to by increased amounts

of both cholesterol and coprostanol per gram dry weight of feces. A previous study assessing the enzymatic activity of cholesterol dehydrogenase in the feces of patients with colon cancer and controls showed a higher level of activity in the patient group, possibly explaining the higher concentration of coprostanol previously observed.[26]

Reddy et al.[24] next investigated the rate of bacterial metabolism of cholesterol in patients with ulcerative colitis since this group of persons is at high risk for developing colon cancer. They compared fecal sterol concentration to a classic control group, relatives of the patients, and a group of persons with other digestive diseases. They found that in those persons with ulcerative colitis total neutral fecal sterol output was significantly greater than that in any of the control groups. This increase was contributed to by increases in the concentration of cholesterol (fourfold) and coprostanol (twofold). When the data were expressed as a ratio of cholesterol to its major metabolites, patients with ulcerative colitis had a significantly higher ratio than any of the control groups, indicating a lower level of bacterial metabolism.

IX. INFLUENCE OF DAIRY PRODUCTS ON INTESTINAL CHOLESTEROL METABOLISM

Interest has been focused on the effect of specific foods on the bacterial metabolism of cholesterol and, going a step further, the implications of this on plasma cholesterol levels. Given that elevated plasma cholesterol has been identified as a major risk factor for coronary heart disease and that nonpharmacological approaches to normalizing the levels are the treatment of choice, the progression is logical.

A number of studies in both animals and humans have looked at the effect of a variety of fermented and nonfermented dairy products on plasma cholesterol. Mann[27] reported that the consumption of both skim and full-fat yogurt significantly decreased plasma cholesterol after the administration of radiolabeled acetate; he attributed the effect to an inhibitor of hydroxymethylglutaryl CoA reductase.

Hepner et al.[28] fed pasteurized and nonpasteurized yogurt and 2% butterfat milk to humans for varying periods of time. They reported that both yogurts significantly lowered plasma cholesterol 5–10% by one week and that this was maintained for 4 weeks, whereas buttermilk had no effect.

Rossouw et al.[29] tested the hypothesis that the "milk factor" proposed by Mann[27] could lower plasma cholesterol in young males. The subjects were fed skim milk, 1.8% fat yogurt, or 3.3% fat milk for 5 weeks. Only the skim milk resulted in a sustained decrease in plasma cholesterol, which the authors attributed to the decreased consumption of saturated fat and cholesterol.

Considering two rat studies, Grunewald[30] reported that feeding fermented acidophilus skim milk, but not unfermented skim milk, for 4 weeks resulted in a decrease in plasma cholesterol. Pulusani and Rao[31] fed skim milk, whole milk, 2% fat buttermilk, yogurt, buttermilk, or sweet acidophilus milk to humans for 3 weeks. No significant differences in plasma cholesterol were observed during that period. Lin et al.[32] conducted a double-blind study to determine the effect of Lactinex, a commercially available tablet containing *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. The viable bacterial count in the preparation was 2×10^6. A total of 354 subjects were entered into the study. There was no difference detected between the treatment and placebo groups for total plasma cholesterol or any of the lipoprotein fractions. The subjects received Lactinex for two 6-week periods separated by a 3-week washout. These results are not

surprising given the very low dose of organisms ingested by the subjects. This dose is several orders of magnitude lower than would be ingested by an individual eating 6-oz. container of yogurt daily.

In an interesting study, Gilliland et al.[33] isolated a strain of *L. acidophilus* selected for its ability to grow in the presence of bile and assimilate cholesterol. Administration of this culture for 10 days to pigs partially prevented a diet-induced elevation in serum cholesterol. Strains that grew in the presence of bile but did not assimilate cholesterol served as a negative control. As a result of these studies Gilliland and Walker[34] looked for a human strain with similar cholesterol-assimilating properties. They reported that an *L. acidophilus*–designated strain NCFM had an appreciable ability to assimilate cholesterol, although not as high as the pig strain, and could be useful for lowering plasma cholesterol in humans.

There are now several studies assessing the efficacy of probiotics in general and two that concentrate on the cholesterol-lowering effects. These have approached the question by using the meta-analysis of existing clinical studies. A major drawback is the lack of studies on specific strains, and therefore the analysis has focused on studies conducted with different strains and products. Two such reviews concentrated on cholesterol-lowering effects.[35,36] The first concluded that no proven cholesterol-lowering effects could be found. The second, focusing only on short-term intervention studies with one yogurt type (one product and specific strain used for producing the yogurt), reported a 4% decrease in total cholesterol and 5% decrease in low-density lipoprotein cholesterol. It is clear that long-term studies are required before any conclusion as to the efficacy of such treatment can be reached.

Bacterial metabolism of cholesterol can be influenced by diet, as evidenced by significant variations among different population groups with different dietary habits. Altered patterns of intestinal bacterial metabolism of cholesterol may place persons at a higher risk for developing the disorder. Additionally, there may to be a relationship between the intake of certain dairy products and plasma cholesterol, although that relationship is far from being defined. More work needs to be done to clarify the relationship between dietary intake, bacterial metabolism, and plasma cholesterol levels.

X. CONCLUSION

The large number of microorganisms (approximately 10^{14}) that occupy the normal human intestinal tract constitute an ecosystem capable of metabolizing a large number of exogenous and endogenous compounds. Among this population the lactobacilli form an active component and participate in many of these reactions. The fate and pharmacokinetics of drugs, procarcinogens, dietary components, and endogenous compounds, such as bile acids, are influenced by the intestinal microflora.

The metabolic events occurring in the intestine have a central role in the fate and regulation of cholesterol in the body. As a consequence of cholesterol's central importance in normal physiology and disease, the role of the intestine in cholesterol metabolism has great significance. A large body of information has been collected regarding intestinal cholesterol metabolism; however, many important details and questions have not been determined or answered, and this area is a fertile ground for future research.

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Human Studies on Probiotics: What is Scientifically Proven Today?

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I. INTRODUCTION

A probiotic has been defined by the ILSI (International Life Sciences Insitute) Europe working group as "a viable microbial food supplement which beneficially influences the health of the host".[1] This definition requires that safety and efficacy of probiotics have to be scientifically demonstrated for each strain and each product. Demonstration of health effects includes research on mechanisms and clinical studies with human subjects. The same ILSI Europe working group also defined probiotic foods as functional if they have been satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction in the risk of diseases.[2] These definitions have set the basis for the assessment of the health promoting potential of probiotics. It has also been used as a basis for the International Dairy Federation Expert Group reports on probiotic health effects.[3,4,5] Based on extensive reviews of data from published reports it can be concluded that specific probiotics have proven

benefits which can be attributed to specific products. These studies and strains are the focus of this discussion.

II. GUT BACTERIA—THE KEY TO HEALTH AND WELL-BEING

The bacteria in our gastrointestinal tract have a profound influence on our well-being. Several recent reviews have addressed the role of gut bacteria and probiotics in human health.[6–8] Living microorganisms have long been used as supplements to restore gut health at times of dysfunction. All of the probiotics used today have been isolated from human gut contents, and healthy subjects have been used as source of probiotic organisms.

III. BACKGROUND FOR ASSESSMENT OF HEALTH EFFECTS

Assessing probiotic efficacy in humans requires one to understand that all probiotic strains are unique and different. Their properties and characteristics should be well defined, and studies on even closely related strains cannot be extrapolated without great caution. It is important to clearly identify each strain using modern methodology and to make all study strains available for all research groups participating in the world-wide assessment work on health effects and mechanisms. There is a tradition of using cultures for fermented milks and other foods to enhance health-related qualities (Table 1).

IV. PROBIOTIC STRAINS AND THEIR EFFECTS: META-ANALYSIS

Several studies have assessed the efficacy of probiotics in general. Several meta-analyses of existing clinical studies have been published. Most assessment studies have focused on the efficacy of probiotics in acute diarrhea in children.[9–13] The objective of these studies was to assess the efficacy of probiotics in general in reducing the duration of acute diarrheal disease in children. The main outcome criteria were differences in diarrhea duration

Table 1 Microorganisms Usually Connected with Beneficial Probiotic and Dietary Properties in Cultured Milks

Bifidobacterium longum
Bifidobacterium bifidum
Bifidobacterium infantis
Bifidobacterium animalis
Bifidobacterium lactis
Pediococcus acidilactici
Lactobacillus delbrueckii subsp. *bulgaricus*
Lactobacillus acidophilus
Lactobacillus casei
Lactobacillus paracasei
Lactobacillus plantarum
Lactobacillus reuteri
Lactobacillus rhamnosus
Propionibacterium freudenreichii
Enterococcus faecium (previous name *Streptococcus faecium*)

or diarrhea frequency between groups. All reviews concluded that probiotic therapy shortens the duration of diarrheal illness in children, but the heterogeneity of clinical studies did not allow firm conclusions on preventing acute gastroenteritis. The second assessment goal was the use of probiotics in antibiotic-associated diarrhea in children. Two studies completed a meta-analysis in this area.[14,15] The reviews concluded that further studies are needed and an assessment of the costs and need for routine use of probiotics should also be conducted.

One review has focused on the use of probiotics and other therapies in ulcerative colitis and Crohn's disease (Baert and Rutgerts, 2000). No firm conclusions on probiotic use could be made.

Two reviews concentrated on cholesterol-lowering effects.[12,16] The first concluded that no proved effects could be found on cholesterol-lowering. The second, focusing on short-term intervention studies with one yogurt type, reported a 4% decrease in total cholesterol and 5% decrease in low-density lipoprotein (LDL) cholesterol. It is clear that long-term studies are required before any conclusion as to cholesterol-lowering effects can be made.

All meta-analyses have been completed assessing studies with different probiotics even though the properties are significantly different and the strain effects cannot be expected to be similar. Thus, future meta-analysis should be completed using studies conducted with the same strain only without combining the data from several strains. This is likely to be possible soon as more studies are published and data become available on single strains or products with defined strain combinations.

A number of reported probiotic health-related effects have been only partially established, but some can be considered reasonably well established and clinically well documented for specific strains. Such strain-specific proven health effects are listed in Table 2. Other reported effects requiring more data before reaching conclusions involving specific probiotic strains are shown in Table 3.

Table 2 Established and Proposed Health Effects of Specific Clearly Defined Probiotics

Scientifically established health effects:
Reduction in duration of rotavirus diarrhea
Reduction in duration of antibiotic-associated diarrhea
Reduction in risk of rotavirus diarrhea
Alleviation of symptoms of lactose intolerance (specific strains only)
Alleviation of symptoms of food allergy in infants
Future challenges to be confirmed in human trials:
Prevention of atopic disease
Reduction in risk of bladder cancer or colon cancer
Alleviation of symptoms of irritable bowel disease and irritable bowel syndrome, Crohn's disease
Reduction in risk of *Clostridium difficile* diarrhea and nutritional management of *Clostridium difficile* colitis
Dietary cholesterol control
Reduction in risk of respiratory infections in infants and children
Reduction in risk of dental caries

Table 3 Current Probiotic Bacteria and Their Reported Effects

Strain	Reported effects in clinical studies	Selected reviews with further references
L. johnsonii LA1	Adherence to human intestinal cells, balances intestinal microbiota, immune enhancement, adjuvant in *H. pylori* treatment	[1,21]
L. acidophilus NCFB 1748	Lowering of fecal enzyme activity, decreasing fecal mutagenicity, prevention of radiotherapy-related diarrhea, improvement of constipation	[1,4,5,44]
L. rhamnosus GG (ATCC 53013)	Treatment and reduction in the risk of rotavirus diarrhea Prevention of antibiotic associated diarrhea Treatment of *relapsing C. difficile* diarrhea Alleviation of atopic aczema in infants Prevention of atopic diseases Reduction in cystic fibrosis symptoms Enhancement of Bifidobacteria microbiota Reduction in *Streptococcus mutans* activity (caries risk reduction)	[4,5,7,12,21,35,37]
L. acidophilus NFCM	Lowering of fecal enzyme activity High lactase activity, treatment of lactose intolerance, production of bacteriocins	[4,5]
L. casei Shirota	Prevention of intestinal disturbances Balancing intestinal bacteria Lowering fecal enzyme activities, positive effects on reducing the recurrence of superficial bladder cancer Reduction in the risk of bladder cancer	[1,4,5,12,21]
S. thermophilus, *L. bulgaricus*	No effect on rotavirus diarrhea No immune enhancing effect during rotavirus diarrhea No effect on fecal enzymes Strain-dependent improvement of lactose intolerance symptoms	[4]
L. acidophilus La-5	Balancing intestinal microbiota, protection against traveler's diarrhea, immune enhancement	[4]
Bifidobacterium lactis Bb-12	Treatment of viral diarrhea including rotavirus diarrhea Balancing intestinal microbiota Reduction in the risk of traveler's diarrhea Treatment of symptoms of food allergy in infants	[4,7,21,33], Saavedra et al., 1996
Lactobacillus gasseri (ADH)	Fecal enzyme reduction, survival in the intestinal tract	[4,5]

(*continued*)

Table 3 *Continued*

Strain	Reported effects in clinical studies	Selected reviews with further references
Lactobacillus reuteri	Colonizing the intestinal tract, shortening of rotavirus diarrhea, balancing intestinal microbiota	[4,12,21,22]
Lactobacillus rhamnosus	Immune enhancement, intestinal microbiota effects	[5]
Lactobacillus rhamnosus DR 10	Immune enhancement, adherence to mucosa, microbiota effects, improvement of the elderly condition	[5,55–57]
Bifidobacterium lactis HN019	Immune enhancement, balancing intestinal microbiota	[5,55–57]
Probiotic combination (VSL 3)	Positive effect in inflammatory bowel disease and irritable bowel syndrome; treatment and prevention of pouchitis, prevention and alleviation of radiotherapy associated diarrhea	[45,46,50], Rembacken et al., 2001, Kim et al., 2002
Escherichia coli NISSL*E*	Positive effect in inflammatory bowel disease	[50], Rembacken et al., 2001
Probiotic mix VSL 3 *(L. bulgaricus, L. plantarum, S. thermophilus*, *B. longum, B. infantis*, and *B. breve)*	Positive effect in irritable bowel disease in adults	[40]
S. boulardii (often registered as a pharmaceutical not in foods)	Prevention of antibiotic-associated diarrhea, treatment of *C. difficile* colitis, alleviation of antibiotic-associated diarrhea	[21]

V. SCIENTIFICALLY PROVEN EFFECTS

A. Lactose Intolerance

There is convincing evidence from several studies that lactose-intolerant individuals suffer fewer symptoms if milk in the diet is replaced with fermented dairy products. The reduced levels of lactose in fermented products, due to partial hydrolysis of lactose during fermentation may contribute to the greater tolerance of yogurt.

The mechanisms of action of lactic acid bacteria (LAB) and fermented dairy products include the following: lower lactose concentration in the fermented product, high lactase activity of bacterial preparations used in the production, and increased active lactase enzyme entering the small intestine with the fermented product or within the viable bacteria able to survive gastric and bile conditions.

The bacterial enzyme β-galactosidase, which can be detected in duodenum and terminal ileum after consumption of viable yogurt, is thought to be the major factor that improves digestibility by the hydrolysis of lactose, mainly in the terminal ileum.

Another factor suggested to influence lactose digestion is the slower gastric emptying of semi-solid milk products such as yogurt.

It is important to assess the β-galactosidase activity of probiotic strains and other LAB used in dairy products. β-Galactosidase activity within probiotics varies significantly from practically nil (e.g., *Lactobacillus* GG) to very high.[4] Thus, both the enzyme activity of the probiotic strain and the activity left in the final product are important for their use in lactose intolerant subjects.

In conclusion, there is good scientific evidence on the alleviation of lactose intolerance symptoms by specific probiotic lactic acid bacteria. However, the strain-specific lactase activities may vary over 100-fold. Thus, different products may have varying lactose contents, and the strains, when released to the duodenum, vary in their lactase activity.[4] Therefore, not all fermented milks are equal in lactose content and microbial lactase activity, and the health effects for lactose-intolerant subjects must be assessed on a case-by-case basis.

B. Rotavirus Diarrhea

Lactobacillus GG has been reported effective in the treatment of rotavirus diarrhea.[17,18] It reduces the duration of diarrhea in about half in children with rotavirus diarrhea. It has also been reported to be effective in the treatment of watery diarrhea in several studies in Asia, with favorable results on colonization.[19] When different LAB were compared for their effects on the immune response to rotavirus in children with acute rotavirus gastroenteritis, differences between various strains were observed. Serum antibodies to rotavirus, total number of immunoglobulin-secreting cells (ISC), and specific antibody-secreting cells (sASC) to rotavirus were measured at the acute stage and at convalescence. Treatment with *Lactobacillus* GG was associated with an enhancement of IgA sASC to rotavirus and serum IgA antibody level at convalescence.[20] It was therefore suggested that certain strains of LAB promote systemic and local immune response to rotavirus, which may be of importance for protective immunity against reinfections. (For reviews on rotavirus diarrhea, see Refs.[1,4,5,7,12].)

The effects of viable and heat-inactivated LAB have been compared in a blinded randomized clinical study. *Lactobacillus* GG administered as a viable preparation during acute rotavirus gastroenteritis resulted in a significant rotavirus-specific IgA response at convalescence. Heat-inactivated *Lactobacillus* GG was clinically as efficient, but the IgA response was not detected. This suggests that viability of the strain is critical in determining the capacity of LAB to induce immune stimulation. Also, in a study with different preparations of LAB using the recommended doses (1.25 g of freeze-dried preparation twice daily for 5 days) in the treatment of rotavirus diarrhea, it was shown that *Lactobacillus* GG (cell concentration 5×10^9 CFU/g) was most effective, while a preparation containing a mixture of *Streptococcus thermophilus* (95%) and *Lactobacillus bulgaricus* (4%) or *L. rhamnosus* (1%) (2.8×10^8 CFU/g) or a preparation containing *L. rhamnosus* (2.2×10^8 CFU/g) did not have a clinical effect on the duration of diarrhea. It has also been reported that *L. reuteri* effectively shortened the duration of watery diarrhea associated with rotavirus (for review, see Refs.[4,12,21]).

Shortened duration of rotavirus diarrhea using *Lactobacillus* GG is perhaps the best-documented probiotic effect. It has been well documented in several studies around the world and in a recent multicenter study in Europe.[18,19]. There are also several studies using heat-inactivated *Lactobacillus acidophilus* LB1 and viable *Bifidobacterium lactis*

Bb-12 and two studies on *Lactobacillus reuterii* reporting shortened duration of rotavirus diarrhea in children.[22] As with other documented effects, it is important to remember that the reported studies are specific to the strains used.

C. Antibiotic-Associated Diarrhea

Antibiotic-associated diarrhea has been prevented by *Lactobacillus* GG in a yogurt form using a dose of two cups of yogurt daily with about 10^7 CFU/mL for adult subjects[23] or as a freeze-dried product using doses varying from 10^9 to 10^{10} CFU/day, with two separate large studies confirming the results in children.[24,25] Thus, it can be concluded that *Lactobacillus* GG is efficient in reducing the risk of antibiotic-associated diarrhea in children.

Black and coworkers[26] reported a double-blind study using 20 healthy volunteers treated with 500 mg of ampicillin divided into two groups. Half of the volunteers received 4×10^9 CFU live lyophilized *Bifidobacterium lactis* and *L. acidophilus* La5. The volunteers receiving lactic acid bacteria were recolonized faster than those receiving placebo and harbored higher counts when compared to the controls. There are also good clinical studies showing the efficacy of *Saccharomyces boulardii* in the treatment of antibiotic-associated diarrhea, and this strain has been approved for pharmaceutical use in some countries, but it is currently not used in foods. Several other LAB strains are currently assessed for their effects in antibiotic-associated diarrhea around the world.

VI. NEED FOR FURTHER ASSESSMENT

A. Allergic Diseases

Reports on the prevalence of atopic diseases (e.g. atopic eczema, allergic rhinoconjunctivitis, and asthma) indicate that such diseases have steadily increased in the industrialized world. Different probiotic *Lactobacillus* and *Bifidobacterium* strains appear to induce distinct and even opposing responses in various models and human studies.[27] However, bifidobacteria appear interesting for microbiota aberrancies related to allergies in infants.[28,29]

The microbiota appears to have a crucial role in allergic diseases. In one prospective study, the intestinal microbiota from 76 infants at high risk of atopic diseases was analyzed at 3 weeks of age by conventional bacterial cultivation and two culture-independent methods.[30,31] Positive skin prick reaction at 12 months was observed in 29% of subjects. At 3 weeks of age, the bacterial cellular fatty acid profile in fecal samples differed between those infants later developing atopic sensitization and those not developing atopy. Fluorescence in situ hydridization was used to show that atopic subjects have more *Clostridium* species and fewer *Bifidobacterium* species in stools compared with nonatopic subjects. Differences in the gut microbiota were suggested to precede the manifestations of atopic disease. This finding was confirmed later in Sweden and Japan, suggesting that microbiota could be a target for prevention studies with probiotics.

Pioneering studies in Finland reported improvement in the clinical course of atopic eczema and cows milk allergy in infants receiving probiotic-supplemented extensively hydrolyzed formula compared to placebo-supplemented formula.[32,33] Similar results have been reported in Japan and in Finland in milk-hypersensitive adults.[34] In these subjects a milk challenge in conjunction with a probiotic strain prevented the immunoinflammatory response characteristic of the response without probiotics.

The preventative potential of probiotics in atopic diseases has been demonstrated in a double-blind, placebo-controlled study.[30,31,35,36] In the first clinical demonstration of probiotics administered pre- and postnatally for 6 months to children at high risk of atopic diseases, the prevalence of atopic eczema was reduced by half compared with infants receiving placebo.[30,31,35,37] An important clinical result was reported after 4-year follow-up, indicating that the effect on risk reduction extends beyond infancy.[29,37]. Additional studies addressing the role of probiotics in the prevention of atopic disease are definitely needed and well warranted based on these first demonstrations. This study reported on both the safety and efficacy of probiotics in allergy prevention, laying the groundwork for future clinical studies. These demonstrations, along with the detection of microbiota aberrancies, can form the basis for designing future functional probiotics for subjects suffering from allergic diseases or at risk of developing allergic diseases.[38]

B. Probiotics, Intestinal Microbiota, and Cancer

A number of studies have focused on the effect of probiotics on intestinal microeclogy and cancer. *Lactobacillus acidophilus*, *Lactobacillus casei* Shirota strain, and *Lactobacillus* GG have been shown to have inhibitory properties on chemically induced tumors in animals.[12,21] Other studies indicate that specific strains of probiotic bacteria may be able downregulate intestinal microbial enzyme activities.[21] This phenomenon may then decrease carcinogen-activating microbial enzymes and has a beneficial effect in the colon, the urinary tract, and the bladder. However, further studies, especially human studies, are needed in this area.

Reports on the benefits of oral administration of probiotic cultured milks and lactic acid bacteria on tumors have been connected with changes related to tumor induction and promotion. The following mechanisms have been indicated in various studies relating lactic acid bacteria intake and cancer:

1. Alteration in intestinal microecology (beneficial microbiota effects)
2. Altered intestinal metabolic activity (decreased conversion of precarcinogens to carcinogens)
3. Normalized intestinal permeability (prevention or delaying of toxin absorption)
4. Enhanced intestinal immunity (enhanced resistance to chemicals, inflammation, and other factors)
5. Strengthened intestinal barrier mechanisms (include some or all of 1–4)

At present, several studies have been reported on cancer and intestinal microecology–related aspects, but the results have not been conclusive and human studies have been missing. The most convincing reports have been published on *Lactobacillus casei* Shirota (Fig. 1). There are several mechanistic studies on the effects of the strain reporting decreased urinary mutagen excretion. Other mechanisms have been assessed in both experimental animals and human subjects. Following the mechanistic studies on decreased urinary mutagen excretion, human clinical studies have been conducted using *Lactobacillus casei* Shirota. In one clinical study and another larger multicenter study the prophylactic effects of oral administration of *Lactobacillus casei* Shirota on the recurrence of superficial bladder cancer have been reported in Japan.[39] Recently, a large Japanese case-control study on the habitual intake of lactic acid bacteria and risk reduction of bladder cancer has been conducted in the specific setting of home delivery of the product. This study suggested that the habitual intake of fermented milk with the strain reduces the

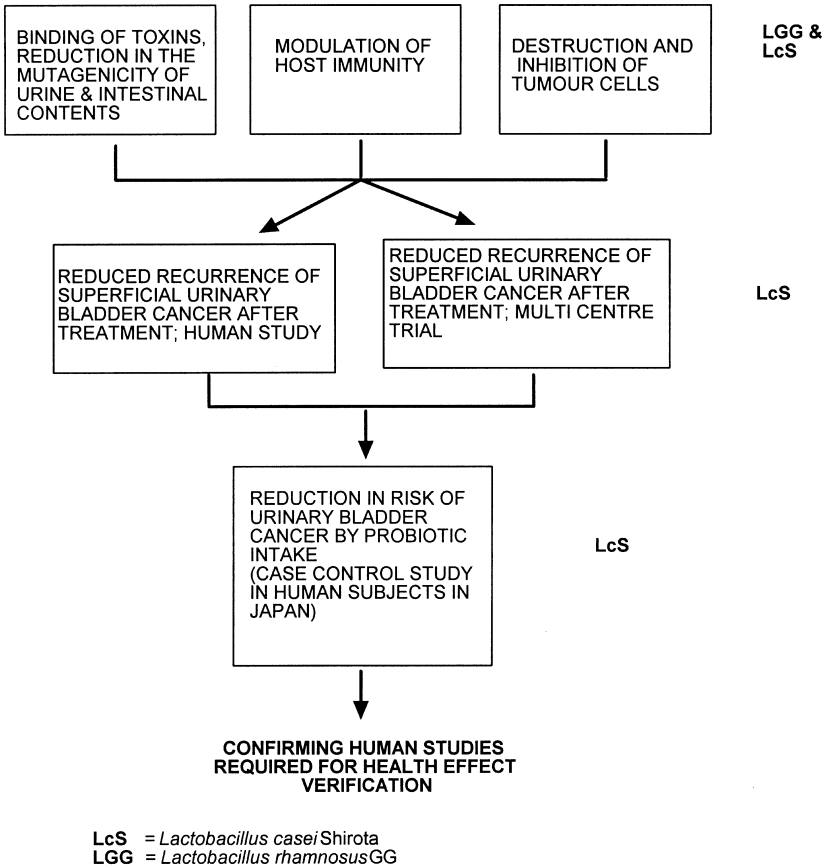

Figure 1 The rationale for specific probiotics (*Lactobacillus casei* shirota and *Lactobacillus rhamnosus* GG) to influence development of events leading to bladder cancer with consequent risk reduction potential.

risk of bladder cancer in the Japanese population. This result combined with mechanistic work and human studies warrants further investigation in other countries.

C. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) has been extensively studied and probiotic products have been assessed as a means of nutritional management of the disease. Among others, *S. faecium* preparations have been evaluated for treatment of IBS patients whose symptoms had been present for an average of 7 years. Although patient-recorded symptoms did not differ significantly in the placebo or *S. faecium* groups, the physician's subjective clinical evaluation of symptoms revealed an significant improvement in the treated group. In more recent studies, reduction of symptoms has been reported with a *Lactobacillus plantarum* preparation. Another study by Brigidi[40] with reported symptom reduction used a probiotic mix VSL-3 (*L. bulgaricus*, *L. plantarum*, *S. thermophilus*, *B. longum*, *B. infantis*, and *B. breve*), and further documentation of symptom relief, including reduction in abdominal bloating in diarrhea-prone IBS patients, was provided by Kim and coworkers.[41] No

reduction in symptoms was reported with *Lactobacillus* GG.[42] There is a rationale for investigating the effect of lactic acid bacteria and cultured milks in the treatment of this common disorder, where intestinal motility and dysfunctions in the intestinal microflora are important factors to consider. Current reports have not clearly proven the benefits, and the strains with the most reported success need to be assessed further. Further human studies with both probiotic bacteria and cultured milks are ongoing in Europe and may provide future strategies for dietary management of this disease.

D. Pouchitis

A probiotic mix has also been used for the alleviation of symptoms of pouchitis. The efficacy of a concentrated probiotic preparation (VSL#3) in the prevention of flare-up in patients with chronic pouchitis was recently documented.[43] More recently it has been demonstrated that administration of VSL#3 is effective in the prevention of the onset of acute pouchitis and improves the quality of life of patients with ileal pouch–anal anastomosis.

E. Radiotherapy Associated Diarrhea

In early reports, *Lactobacillus acidophilus* and other LAB have had a positive effect on radiotherapy-induced diarrhea.[44] There has been renewed interest in the nutritional management of this disorder, and promising new results have been reported using the probiotic mix VSL#3.[45,46] Clearly, when the intestinal microbiota aberrancies in this type of diarrhea are identified as targets for probiotic treatment, more specific species or species combinations can be administered to prevent the diarrhea or to alleviate symptoms.

F. Inflammatory Bowel Disease

Inflammatory bowl disease (IBD) is a term usually covering two related conditions: ulcerative colitis (UC) and Crohn's disease. Both are chronic inflammatory conditions of the intestinal tract which are common in the western world. Symptoms vary from persistent diarrhea to constipation, and the symptoms have been the target of probiotic trials. An increasing number of clinical and experimental studies demonstrate the importance of intestinal microbiota in these diseases. Probiotic bacteria may counteract the inflammatory process by stabilizing the gut microbial environment and the intestine's permeability barrier and by stimulating the microbiota, enhancing the degradation of enteral antigens and altering their absorption and immunogenicity.

It is apparent that probiotics can be useful in the dietary management of some forms of IBD, but they do not replace pharmaceutical treatment. The basis for probiotic treatment has been summarized by Shanahan[47]. Several studies using *Lactobacillus* GG, *Lactobacillus salivarius* UCC118, or *E. coli* Nissle 1917 have reported benefits in reduced use of pharmaceuticals, improved quality of life, and reduced relapses.[48–50] In adults operated upon for the condition, however, *Lactobacillus* GG failed to prevent recurrence during one year of follow-up.[51] A recent study provides evidence for successful treatment with a nonpathogenic, potentially probiotic strain of *E. coli* in maintaining remission in ulcerative colitis.[52] Studies with *E coli* have been promising, and further assessment of the preparation is underway.

It is important to characterize the intestinal microbiota aberrancies (both intestinal content and mucosal microbiota) and conduct more human studies to define the effects

of specific probiotic strains on each form of inflammatory bowel disease. The potential of several probiotic strains clearly warrant assessment.

G. Traveler's Diarrhea

Several studies on the prevention of traveler's diarrhea show positive outcome for *Lactobacillus* GG and a combination of *Lactobacillus acidophilus* LA5 with *Bifidobacterium lactis* Bb-12 (for review, see Refs.[1,4,21]). These studies show some indications of beneficial effects, and some studies reported no effects, but information from large human studies using defined strains on traveler's diarrhea is still largely lacking. The current data on traveler's diarrhea does not show consistent scientifically proven effects for any strains used (Table 4). Thus, further human studies with known bacterial etiology diarrhea should be conducted to verify the earlier results.

Table 4 The Efficacy of Prophylactic *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* Preparation in Preventing Traveler's Diarrhea (TD)

Preparation	No. of cases	TD present	TD absent	Protection rate
Lactobacillus [a]				
2×10^9	154	82	72	n.s.
Placebo	165	78	87	n.s.
S. faecium SF 68				
1.5×10^7	672	234	438	n.s.
Placebo	652	248	404	n.s.
1×10^9	401	188	213	n.s.
Placebo	419	210	209	n.s.
Lactobacillus GG[b]				
10^9 cfu/day	153	68	85	8.0
Placebo	178	74	104	n.s.
Lactobacillus GG[c]				
10^9 cfu/day	71	17	54	39.5
Placebo	75	30	45	($p < 0.05$)
Probiotic mix[d]				
10^9 cfu/day	40	17	23	39.4
Placebo	41	29	12	($p < 0.05$)
Lactobacillus GG[e]				
2×10^9 cfu/d				($p < 0.05$)
Placebo	245	na	na	47 %
L. fermentum KLD[f]				
10^{11} cfu/day	80	19	64	n.s.
L. acidophilus (LA)				
10^{11} cfu/day	101	26	75	n.s.
Placebo	101	24	77	

[a]Austrian tourists (Kollaritsch and Wiedermann, 1990).
[b]Marmaris/1 week (Oksanen et al., 1990).
[c]Alanya/1 week (Oksanen et al., 1990).
[d]Egypt (Black et al., 1989).
[e]American tourists (Hilton et al., 1996).
[f]British soldiers (Katalaris et al., 1995).

H. Helicobacter pylori

Specific strains of LAB have been reported to inhibit many intestinal pathogens including *Helicobacter pylori.* Lactic acid bacteria are often able to survive the acidic gastric conditions, and therefore it has been proposed that they may have a beneficial influence during the eradication of *Helicobacter pylori*, which is involved in the process of gastric ulcer development. It has been reported that both the inhibitory substances and the specific strains may influence the survival of *Helicobacte*r, and studies have been conducted especially with *Lactobacillus johnsonii*. It has been shown that there is good in vitro inhibition and that fermented milk containing the strain has a positive effect when consumed during *Helicobacter* eradication therapy.[53] However, more controlled human studies conducted in different populations need be conducted to verify this effect.

VII. FUTURE CHALLENGES

There is a need to further develop and conduct clinical studies with probiotic bacteria since only a few of the claimed effects are hypothesis-based and backed by good clinical studies. It is important to make sure that the nutritional and human studies are well defined and planned. Each strain and product should be documented and tested independently, since extrapolation of data from closely related strains is not acceptable. Table 3 contains suggestions for scientific documentation of health effects in human studies. The most important task in formulating probiotic preparations is to carefully identify the targets for which they are used. Second, the preparations for each target should be specifically identified, as each probiotic has a distinct property profile and may be effective in one target and not in

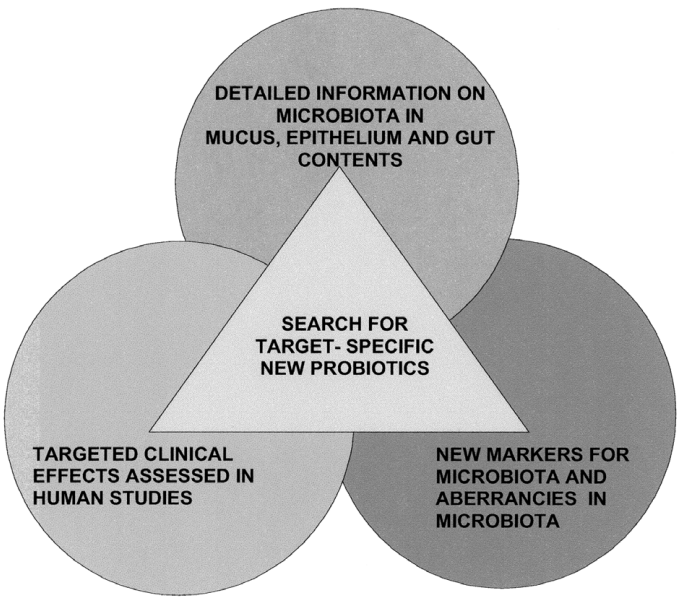

Figure 2 Suggested areas for consideration when selecting new target-specific probiotics for human use.

Table 5 Requirements for Nutritional and Clinical[a] Studies of Functional Foods and Probiotic Foods with Health Claims

Strains clearly identified and stored in accessible culture collections
Strain characteristics clearly documented
Each strain documented and tested independently
Extrapolation of data from closely related strains not acceptable
Well-defined probiotic strains, well-defined study products, well-defined study populations
Double-blind, placebo-controlled and randomized human studies
Results confirmed by several independent research groups[b]
Publication in peer-reviewed journals

[a]Clinical studies conducted according to the good clinical practice in human subjects
[b]Clinical results should be confirmed by several independent—at least two—research groups

another. This may require revising the current selection criteria for probiotics to select for target and site-specific future strains for particular uses to improve human health and well-being.[64,65] The tasks involved in new and novel selection criteria are described in Figure 2.

Protocols for human nutrition studies need to be developed for probiotics and functional foods. It is clear that the requirements set by ILSI Europe working groups form the minimum basis for assessment of clinical efficacy of probiotics in humans.[1,2] In some cases postmarketing surveillance studies on intakes and long-term effects are desirable. Such studies have also been used for the safety assessment of current probiotics.

The design of clinical studies used in pharmaceutical development should serve as a reference point, but specific protocols and specific criteria relevant to functional foods may also be needed. It is necessary to identify specific target groups consisting of individuals who may present higher/lower susceptibilities to potential adverse effects. It is important to clarify the long-term consequences of the interactions between functional food components and functions in the human gastrointestinal tract. The interactions between probiotic components, other food materials, and pharmaceutical preparations must be carefully monitored (Table 5).

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19

Safety of Novel Probiotic Bacteria

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I. PROBIOTICS PAST AND FUTURE

Maintenance of normal intestinal flora and improved digestion are benefits generally ascribed to existing probiotics. Different bacterial strains are being investigated for their therapeutic potential to treat inflammatory bowel disease, inhibit pathogenic bacteria, ameliorate diarrhea of various etiologies, prevent dental caries, and reduce allergy. It is postulated that probiotic bacteria have a role in immune modulation and suppression of carcinogenesis. While research has concentrated on probiotics to modulate intestinal flora, recent studies have sought to restore the vaginal and urinary ecology, either indirectly after ingestion or by topical application.[1]

Probiotic organisms are commonly from the genera *Lactobacillus* and *Bifidobacterium*, strains of *Enterococcus* and *Saccharomyces* species being among the exceptions. Members of these genera are generally regarded as safe because they have a long history of use as dairy starters, which, unlike probiotics, are not specifically adapted to survive in the gastrointestinal tract. They have rarely been associated with disease, usually as opportunistic infections in people with predisposing conditions.[2,3]

Many of the organisms to which we ascribe probiotic effects have had their origins in dairy products and the manufacture of fermented foods. Thus they have been consumed as constituents of these products for centuries with no apparent adverse effects. Probiotics consumed in foods and dietary supplements are accorded a generally recognized as safe status (GRAS) and do not have to comply with more rigorous guidelines for probiotics, which claim amelioration or prevention of disease in clinical applications. "History of safe use" as a criterion for the safety of food organisms is an arbitrary classification, and food organisms claiming this status have not been defined previously.

To redress this, the International Dairy Federation and the European Food and Feed Cultures Association have jointly initiated a referenced inventory of microorganisms with

a documented history of use in food manufacture, i.e., starter cultures.[4,5] The inventory lists taxonomy and applications for lactic acid bacteria, *Enterococcus* and *Streptococcus* species, yeasts, and molds. It is an evolving document that will be modified as candidate organisms are identified, added, removed, or reclassified with changes in taxonomy. This initiative is a systematic attempt to catalogue organisms that may reasonably be expected to be safe because of their consumption in foods without apparent ill effect. It provides a basis for genera, species, and strains to be identified as safe and a potential source of new probiotic organisms.

New species and more specific strains of probiotic bacteria are constantly being sought for novel probiotic products. The safety status of novel organisms intended for probiotic use cannot be assumed. Prior to the incorporation of novel strains into products, their efficacy should be carefully assessed and an evaluation made as to whether they share the safety status of traditional food-grade organisms.

The concept of genetic manipulation of bacteria for a specific probiotic function is appealing. Consumer resistance to genetically modified organisms (GMO) in foods is such that GMO probiotics are unlikely to be used in the near future, with the possible exception of clinical applications. Steidler et al.[6] showed that a probiotic *Lactococcus lactis* genetically engineered to secrete cytokine interleukin (IL)-10 prevented colitis in a mouse model of colitis. A recombinant strain of *Bacillus subtilis* 2335 has been designed which produces proteins with antibacterial and antiviral properties shown to enhance the effectiveness of antitumor therapy in mice.[7] Probiotics can thus be designed to produce potent bioactive chemicals. Extrapolation from proof of principle in a murine model to development of therapeutic applications for humans demands a stringent safety assessment of such GMO probiotics.

The demonstration of efficacy in probiotics offers vast opportunities for the development of human and veterinary products. The addition of novel bacterial strains to foods and therapeutic products requires reconsideration of the procedures for safety assessment. Probiotic products that claim specific nutritional, functional or therapeutic characteristics blur the boundaries between what is a food, a diet supplement, or a medicine, posing challenges for regulators.

Evidence for the safety and efficacy of probiotic organisms has until recently been largely anecdotal or based on relatively little and often poorly designed research. Lactic acid bacteria (LAB) and yeasts intrinsic to the production of traditional foods have been accepted as safe without any real scientific criteria, partly because they exist as normal commensal flora, and because of their presence for generations presumably without adverse effect.

The introduction of a new probiotic culture demands that it be at least as safe as its conventional counterparts. Suggested safety criteria have included but not been limited to unequivocal identification of species and strain, with candidate strains lodged in culture collections for reference and comparison; a profile of intrinsic properties of the organism such as metabolic and enzyme activities, antibiotic resistance and the potential for its transference; host-specific behavior of the strain; and host factors predisposing to infection.[3,8]

II. UNEQUIVOCAL IDENTIFICATION

For many consumers the term probiotic is a new concept and they are reliant on the manufacturer's label for appropriate information. The consumer is entitled to expect

that the label on a probiotic product accurately reflects its contents, i.e., the organism is what it purports to be, it is present alive in a specified concentration range for a stated period, and that the suggested serving size contains sufficient organisms to achieve the claimed benefit.

The safety of a putative novel probiotic strain is contingent on its accurate identification. Is the strain associated with safe food use, an intestinal strain isolated from humans, a strain isolated from animals, or a genetically modified strain? Because probiotic effects are known to be strain specific, unequivocal identification of the probiotic bacteria at the genus, species, and strain level is essential. Correct taxonomic identification of both species and strain is a safety issue for quality control of the product, consumer or prescriber information, diagnosis and appropriate treatment of suspected clinical cases, and epidemiological surveillance of the exposed population.

Combinations of phenotypic and molecular techniques are available to identify species and discriminate between strains. Closely related strains may be differentiated by molecular typing techniques such as DNA fingerprinting by pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), ribotyping, restriction enzyme analysis, and plasmid profiling. Borriello et al.[9] suggest that because of the infrequency of probiotic bacteremia and the sophisticated methods and experience needed, identification and confirmation of species and strain characteristics of suspect clinical isolates should be referred to national reference centers.

Chemometric methods for pattern recognition incorporating biological and morphological measurements are being developed for automated classification of microorganisms. Characterization of microorganisms by molecular spectroscopy involves analysis of a culture, usually during the growth phase, by Fourier transform mid-infrared spectroscopy. Sophisticated pattern recognition techniques offer the potential for non-invasive, rapid classification of microbial organisms and metabolite patterns from small samples.[10]

III. TAXONOMY

The taxonomy of lactic acid and other bacteria has changed significantly with the advent of genetic methods of classification. Strains previously thought to be dissimilar have merged, while other strains have been added or reassigned to different genera. The persistent use of incorrect or nonexistent species names on product labels despite taxonomic reassignation is an issue for the safety and credibility of probiotics. Inaccurate nomenclature has no scientific or regulatory validity, misinforms or confuses the consumer, and compromises the safety of the product.

Yeung et al.[11] used partial 16S rDNA sequencing to identify named commercial strains obtained directly from the manufacturer and found discrepancies in 14 of 29 species designations. Lourens-Hattingh and Viljoen[12] concluded that probiotic cultures in South African yogurt were little more than a marketing tool upon finding that the initial counts of *Bifidobacterium bifidum* in three different sources of commercial yogurts were lower than 10^6 cfu/mL and thus below the therapeutic minimum. Weese[13] identified isolates from eight veterinary and five human probiotics to find accurate descriptions of organisms and concentrations for only two of the 13 products.

Temmerman et al.[14] found that of isolates from 55 European probiotic products, 47% of food supplements and 40% of dairy products were mislabeled. The food supplements yielded either no viable bacteria (37%) or significantly lower counts than the

dairy products, contradicting the concept that health benefits derive from the presence of a minimum concentration of live probiotic bacteria.

In 6 products, all species isolated conformed to the label description; in 19 products they differed from those listed. *Enterococcus faecium*, followed by *Lactobacillus rhamnosis*, was found most frequently in food supplements. *E. faecium* was isolated in such high numbers that contamination was unlikely to be the source. Two of the 22 food supplements purporting to contain *Lactobacillus acidophilus* did. Bifidobacteria were isolated from 5 of 27 products claiming to contain them, despite the use of different selective media. The organism most frequently claimed to be in and isolated from dairy products was *L. acidophilus*, though it was not necessarily found where claimed.

These studies corroborate previous findings by Hamilton-Miller and Shah[15] and Playne[16] and demonstrate continued inaccurate identification and mislabeling of probiotic products.

IV. ENTEROCCOCI AS PROBIOTICS

Enterococci are commensal microbiota of the gastrointestinal tract, occur naturally in some foods, and are common in veterinary probiotics. They are not typical LAB. Several species are pathogens and have been isolated in nosocomial and other infections, often in pure culture. Some enterococci demonstrate resistance to antibiotics, including vancomycin, and have the ability to transfer antibiotic resistances.[3] With the exception of strains such as *E. faecium* and *Enterococcus faecalis*, they are not recognized as safe based on a history of use.[17]

Lund et al.[18] evaluated the ability of viable *E. faecium* to survive gastrointestinal transit in a prospective, randomized blind study of 20 healthy volunteers who had not consumed antibiotics in the 3 months prior or probiotics in the previous 30 days. For 10 days volunteers consumed 150 mL/day of fermented milk product containing Causidio® culture, a mixture of two strains of *Streptococcus thermophilus* and one of *E. faecium*, equivalent to a daily dose of $4.5-7.5 \times 10^9$ colony-forming units (CFU) of *E. faecium.* Half of the subjects were treated simultaneously with vancomycin (12 mg four times daily) for 10 days. Isolation and identification of *E. faecium* from fecal samples was undertaken at days 0, 10, and 31 (21 days after treatment ceased) by PFGE and phenotypic analyses.

In subjects given probiotic alone, the amount of probiotic *E. faecium* recovered from feces at day 10 of intake compared with total *E. faecium* ranged from 100% (3 subjects) to 13–52% (3 subjects) and $<2\%$ (4 subjects). The strain was not detected 3 weeks after intake ceased, suggesting that it does not persist. Probiotic *E. faecium* was not detected at day 10 in vancomycin-treated subjects, probably because this strain is vancomycin sensitive and colonization was prevented. An unexpected finding was that total *E. faecium* numbers in day 31 fecal samples were increased compared to day 0 levels, but the isolates were not closely related to either the probiotic or preexisting strains.

For some subjects the probiotic strain was the predominant strain of *E. faecium.* This strain has previously been shown by the authors to acquire the *van*A cluster gene for vancomycin resistance in vitro.[19] Although its in vivo ability to transfer antibiotic resistance by conjugation has not been ascertained, it would be prudent to consider that in vivo conjugation in a safety evaluation.

The intrinsic capacity of *Enterococcus* species for survival and the pathogenicity of some species, coupled with their tendency to exchange genetic material and acquire antibiotic resistance determinants, renders their use as probiotics questionable.

V. SPORE-FORMING PROBIOTICS

Novel probiotics may be from other genera of the LAB group, e.g., *Lactococcus* or *Leuconostoc*, or from food-associated genera such as *Propionibacterium.* Other genera and species such as *Clostridium* and *Bacillus* are proposed as potential probiotics.[3]

Bacillus species are spore-forming bacteria, ubiquitous in the environment and considered to have low pathogenicity. They do not colonize the human intestine but have a transient presence in ingested foods. *Bacillus clausii* (previously classified as *B. subtilis* species, a constituent of the probiotic Enterogermina®) is a mixture of spore forms of strains of *B. subtilis* given orally as a pharmaceutical probiotic.

Spinosa et al.[20] inoculated two groups of BALB/c mice intragastrically with a single dose of 10^9 spores of either *B. clausii* (Enterogermina) or a *B. subtilis* MO1099 derivative. Samples were taken from intestinal sites, lymph organs, and blood at 4, 24, and 72 hours after inoculation. Spores of the inoculated *Bacillus* strains were found at all intestinal sites at 4 hours. They were largely excreted in the feces as spores, with their presence decreasing exponentially over 72 hours, at which time they comprised a tenth of the total spore count. The minimum inhibitory concentration of conjugated bile salt taurodeoxycholic acid for the *Bacillus* strains was 100- to 1000-fold lower than that of normal human intestinal bacteria, a possible explanation for the lack of spore germination and intestinal growth.

Bacteria and spores were not detected at significant levels in blood. In one mouse *B. clausii* was detected in the mesenteric lymph nodes and spleen with a cumulative count of spores and vegetative cells 10-fold higher than the spore count alone (1.4×10^4 vs. 1.6×10^3 CFU/g and 2.0×10^4 vs. 3.6×10^3 CFU/g, respectively).

The observation that spores were unable to germinate in the murine intestinal tract, but grew vegetatively after translocation underlines the importance of elucidating those factors favorable for translocation of spore-forming organisms and their subsequent germination external to the gastrointestinal tract, particularly where the genus may have pathogenic members.

Oggioni et al.[21] reported blood cultures positive for *Bacillus* in an immunocompromised patient previously treated with Enterogermina. The two different strains isolated were identical to two from the pharmaceutical product and exhibited multiple antibiotic resistance. It is possible that *B. subtilis* infections are underreported and their significance unrecognized because of their status as a contaminant organism. Further, Oggioni et al.[21] notes that *Bacillus* infection as a cause of death is currently not represented in World Health Organization (WHO) statistics.

Mechanisms of resistance have been studied in *Clostridia* for the β-lactamases. Resistance to penicillin is especially common in *C. butyricum. C. butyricum* produces β-lactamases, which are inducible by some β-lactam antibiotics such as cephalothin, cefoxitin or moxalactam but not penicillins, and inhibited by sulbactam.

Rigorously designed studies are needed to characterize and demonstrate the efficacy and safety of spore-forming probiotic bacteria.

VI. PROBIOTICS IN ANIMALS

The use of probiotics in food animals and aquaculture is well established. Probiotics are reported to improve general health, increase growth and weight gain, and suppress pathogens. Overuse of antibiotics in animal husbandry and the possibility of antibiotic

resistance have heightened interest in probiotics as alternatives. It has been suggested that the use of probiotics in food animals can reduce the risk of pathogen transfer from food to humans.[22] Conversely, transferable antibiotic resistance determinants from strains of animal probiotics potentially harboring these genes may also in theory enter the human food chain.[3]

Aarestrup et al.[23] tested *E. faecalis* and *E. faecium* isolated from humans, chickens, and pigs for susceptibility to 12 different microbial agents and the presence of genes encoding resistance using PCR. The same combinations of antibiotic resistance were observed among isolates from human and animal origin, and the same genes encoding resistance were detected. The frequent detection of similar resistance patterns and genes indicated that transmission of resistant enterococci or resistance genes occurs between humans, chickens, and pigs.

The European Commission (EC)[24] Scientific Committee on Animal Nutrition (2003) has recommended that, before a bacterial strain can be accepted as an animal probiotic, the intrinsic or acquired genetic basis of observed resistance to representative antibiotics and its transferability should be determined. For species with known intrinsic resistance to an antibiotic, such as vancomycin resistance in lactobacillus, the absence of known resistance genes should be confirmed. Strains carrying acquired resistance to antibiotics used in veterinary or human medicine should not be used in microbial feed additives, except when the basis of resistance is a mutation on a gene intrinsic to that bacterium. This policy would exclude from use any probiotic feed additive containing one or more bacteria with resistance genes capable of being transferred to other bacteria. It is also more restrictive than conditions applying to probiotics for human consumption.

The fact that probiotic feed additives are used in agriculture and aquaculture does not obviate the need for them to be safe for humans. Fish and animal probiotics have the potential to cross into the human food chain or transfer antibiotic resistance, and thus should be demonstrated as safe in animals and humans.

Adhesion to intestinal mucosa by probiotic bacteria is thought to be a significant mechanism by which they exclude pathogens. Rinkinen et al.[25] evaluated the in vitro ability of LAB strains to inhibit adhesion of canine and zoonotic pathogens to canine small intestinal mucus. Unexpectedly, *E. faecium* M74 and *E. faecium* SF273 both significantly enhanced the percentage adhesion of *Campylobacter jejuni* to $134.6\% \pm 17.4$ and $205.5\% \pm 75.0$, respectively, of the control without LAB ($p < 0.05$). Companion animals are thought to be a reservoir of *C. jejuni.* Many veterinary probiotics for the treatment of canine intestinal disorders contain *Enterococcus* species. The observation that *E. faecium* enhanced rather than excluded the adhesion of *C. jejuni* suggests that *E. faecium* may be a risk factor in human *Campylobacter* infection.

VII. PROBIOTICS AND INFECTION

It is essential that a probiotic should not have the ability to invade the host cells and cause infection. What is its pathogenic potential? Do other strains or related species cause clinically important infections or produce toxins? Probiotic organisms must be sensitive to broad-spectrum and commonly used antibiotics. This is a significant issue where the intestinal barrier is immature, as in infants; where its integrity is impaired from radiotherapy, antibiotic treatment, or disease; and in immunocompromised states, such as human immunodeficiency virus (HIV) infection. With advances in medical care, an increasing

proportion of the community may be immunocompromised at some time or at risk of opportunistic infection.

Wolf et al.[26] undertook a double-blind, placebo-controlled study assessing the safety of probiotic *Lactobacillus reuteri* in HIV adults and found the organism to be well tolerated with no significant safety problems. In a review of probiotic safety, Borriello et al.[9] found no published evidence that immunocompromised patients had an increased risk of opportunistic infection from probiotic lactobacilli or bifidobacteria.

Two clinical cases have been reported in which a lactobacillus indistinguishable from an ingested probiotic strain has been identified in association with infection. A 74-year-old woman with hypertension and diabetes mellitus developed a liver abscess in association with pneumonia and pleural empyema. She had a history of drinking a probiotic milk containing *L. rhamnosus* GG, and a strain indistinguishable from that was isolated from the abscess.[27] A 67-year-old man with mild mitral regurgitation developed endocarditis after dental extractions. His blood cultures were positive for a strain of *L. rhamnosus* indistinguishable from that in the probiotic capsules he chewed.[28]

A valuable adjunct to future epidemiological studies, such as that by Salminen et al.[29] (see Sec. XI), would be an analysis of what relationship, if any, may exist between the clinical status of the patient and the presence of *Lactobacillus* bacteremia.

Lactobacillus species in general are thought to have low pathogenicity or be opportunistic pathogens in immunocompromised individuals or those with serious underlying disease. It has been suggested that *L. rhamnosus* in particular warrants surveillance because it is associated with more lactobacillemias than other lactobacilli. *L. rhamnosus* is among the most common *Lactobacillus* species in the human intestine, so this may be relative to its extensive presence in the intestine.[30]

VIII. ANTIBIOTIC RESISTANCE

The potential exists for viable probiotics to colonize the intestinal tract and transfer genetic material. Whether resistance genes can be transferred by a probiotic organism to the endogenous flora, or vice versa, and the impact this would have on antibiotic treatment has yet to be elucidated.

In aerobic bacteria conjugation mediated by plasmids or R factors has been documented as the most widespread system for transfer of antibiotic resistance. Antibiotic resistance mechanisms, their genetic nature, and transfer characteristics of resistance determinants have been studied comparatively recently in anaerobic bacteria. It has been shown that the plasmid that encodes for macrolide resistance can be transferred from *L. reuteri* to *E. faecium* and from *E. faecium* to *E. faecalis* in the mouse gastrointestinal tract.[2]

Lactic acid bacteria are naturally resistant to many antibiotics by virtue of their structure or physiology. In most cases the resistance is not transferable and the species are also sensitive to antibiotics in clinical use. However, it is possible for plasmid-associated antibiotic resistance to spread to other species and genera. The transmissible resistance of enterococci to glycopeptide antibiotics such as vancomycin and teicoplanin is of particular concern, as vancomycin is one of the remaining effective antibiotics for the treatment of multidrug-resistant pathogens.[30]

The study by Temmerman et al.[14] found that 68.4% of probiotic isolates were resistant to two or more antibiotics. Strains of lactobacilli were found resistant to kanamycin (81%), tetracycline (29.5%), erythromycin (12%), and chloramphenicol (8.5%).

The disc diffusion method showed 38% of *E. faecium* isolates were resistant to vancomycin, while the PCR-based *van* gene detection assay showed they were susceptible.

The potential for gene transfer is difficult to assess in vivo. It is also difficult to assess what level of gene transfer, if any, may be considered acceptable by the community. It is a significant reason to select strains lacking the potential to transfer genetic determinants of antibiotic resistance. There is little basis for scientific regulation of strains with intrinsic resistance, as little is known about the levels of intrinsic resistance in current probiotic and food strains. Systematic screening for antibiotic resistance in probiotic strains is not undertaken at present.

IX. WHEN IS SOON ENOUGH FOR PROBIOTICS?

The colonization of the sterile neonate gut commences at birth in a gradual process modulated by factors such as the surroundings, gastrointestinal disease, antibiotic use, and diet. The pattern of bacterial colonization differs between premature and full-term infants and with the manner of their delivery.[7] The first bacteria originate from maternal gut flora, but cesarean-born babies are colonized more by the surrounding hospital bacteria. After the first week, bifidobacteria, bacteroides, clostridia, enterobacteria, and streptococci are the dominant flora.[31]

In breast-fed infants specific strains of bifidobacteria predominate, while formula-fed babies have a complex profile of microorganisms similar to adults, with enterobacteria, lactobacilli, bacteroides, clostridia, bifidobacteria, and streptococci. After weaning, the microbiota resemble that of adults. Bifidobacteria predominate from infancy through to old age, but their numbers decline with age.[32]

The gut microbiota are the major source of microbial stimulus in infancy. The initial colonization by and composition of the gut microbiota are pivotal to the development of immune responses and normal gut barrier function. Healthy individuals are tolerant of their own gut microbiota, but tolerance is impaired in patients with inflammatory and allergic diseases,[31] resulting in altered microbiota as the normal flora respond to inflammatory processes in the gut or elsewhere. A study by Kalliomäki et al.[33] indicates that the composition of gut microbiota differs between healthy and allergic infants.

Recent data showed differences in the *Bifidobacterium* population and immunoregulatory potential between healthy and allergic infants.[34–36] Christensen et al.[36] showed that different *Lactobacillus* species have very different activation effects on the gut cells that initiate local immune responses. The nature of the immune response varied with probiotic strain and was strongly influenced by the dose.

Kalliomäki et al.[33] tested whether *L. rhamnosus* GG could prevent the occurrence of atopic eczema in at-risk infants in a randomized double-blind, placebo-controlled trial. *L. rhamnosus* GG was given to mothers prenatally for 2 weeks before delivery and 6 months postnatally if breast feeding, or to the infant if not. The incidence of atopic eczema in the first 2 years of life was halved compared to that in infants given placebo. This study showed that a specific strain of probiotic bacteria strongly influenced immune regulation in infants.

One putative mechanism underlying the basis of therapy by specific strains of probiotic bacteria is normalization of the balance between generation of pro-and anti-inflammatory cytokines. Mechanisms by which a probiotic actually prevents allergy are yet to be elucidated, but are thought to involve the generation of anti-inflammatory cytokines.[32]

This raises significant questions about the use of probiotics in infancy. The long-term effects of probiotics on the composition of the gut flora and gut immunity during maturation are not known. Once a probiotic strain is incorporated into the normal microbiota, as has been documented during infancy, the potential to stimulate an immune response may be abolished with a consequent loss of probiotic potential.

The strain-specific properties of a probiotic need to first be characterized by in vitro testing of the immunomodulatory effects. It is known that not all *Lactobacillus* and *Bifidobacterium* strains have beneficial effects. Molecular factors modulating immunoregulation will need to be elucidated. Immunological effects will require assessment in specific at-risk populations, as the response of "normal" gut microbiota to probiotic intervention varies with age and the clinical status of the subject. Safety evaluation of long-term health effects will be important in the selection of, and characterization studies for, a probiotic.

X. CLINICAL STUDIES

Clinical studies in humans have investigated the effect of oral administration of probiotics on the balance of intestinal microbiota and in a variety of disorders. Until recently many studies were of inadequate design and produced unreliable data. Features of inadequate studies include: (a) absence of a patient control group; (b) small treatment groups; (c) undefined treatment groups; (d) a wide age range within a treatment group; (e) a diversity of antibiotic treatments; (f) an absence of dosing criteria such as dose and duration; or (g) subjects with symptoms of concurrent disease with the potential to confound an observation of adverse effects. The gold standard is a controlled study with randomized, blind assignation to treatment, placebo, and untreated groups.

The safety of a probiotic is inextricably linked to its efficacy. The design of clinical trials for a novel probiotic would ideally provide evidence of its presence, persistence, or colonization in the treated groups compared to placebo groups before, during, and after treatment. The number of organisms and their viability in the product at the onset and conclusion of the clinical trial should also be enumerated.

XI. EPIDEMIOLOGICAL SURVEILLANCE

Two Finnish studies have investigated the incidence of infections associated with LAB. In the first study 16S rRNA methods were used to characterize and identify LAB isolated from blood cultures of bacteremic patients in southern Finland.[37] The total number of infections caused by lactobacilli was extremely low, and the probiotic strain newly introduced in fermented milks was not associated with infections.

In a subsequent study, lactobacilli isolated from bacteremic patients between 1989 and 1994 were compared to common dairy or pharmaceutical strains.[38] From a total of 5192 blood cultures, 12 were positive for lactobacilli, an incidence of 0.23%. None of the clinical cases could be related to lactobacilli strains used by the dairy industry. In both studies, patients with lactic acid bacteria bacteremia had other severe underlying illnesses.

In a recent study, Salminen et al.[29] examined the incidence of lactobacilli bacteremia in the Finnish population for the period corresponding to a rapid increase in consumption of the probiotic strain *L. rhamnosus* GG (ATCC 53103). This strain was isolated from human intestinal flora and introduced into dairy products in 1990. By 1999 the annual per capita consumption was estimated at 6 L (3×10^{11} CFU) per person per year.

The Helsinki University Central Hospital collected all *Lactobacillus* isolates from blood cultures and cerebrospinal fluid in its catchment area from 1990 to 2000. Blood culture isolates were also collected for all cases of *Lactobacillus* bacteremia reported (and unreported) by mandatory notification to the National Infectious Disease Register from its inception in 1995 to 2000. Species were characterized and compared to *L. rhamnosus* GG strain by molecular epidemiological methods.

Ninety cases of *Lactobacillus* bacteremia were identified between 1995 and 2000, when the population in Finland was 5.2 million. Of the 66 isolates available for species-level identification, 48 were *Lactobacillus* isolates, with the most common species being *L. rhamnosus* (26, 54%), *L. fermentum* (9, 19%) and *L. casei* (7, 15%), respectively. In 35 cases more than one bacterial species other than *Lactobacillus* was also identified. Eighteen of the 66 isolates (27%) were organisms other than *Lactobacillus.* Eleven of the 26 *L. rhamnosus* strains were indistinguishable by PFGE from the probiotic *L. rhamnosus* GG.

No increase in the incidence or proportion of *Lactobacillus* bacteremia was observed, despite a clear increase in the number of cases of bacteremia over the period. *Lactobacillus* isolates comprised 0.24% of all blood culture isolates, consistent with previous Finnish reports.[38] The average annual national incidence of *Lactobacillus* bacteremia was estimated as 0.29/100,000 people per year. The study provides evidence that the increased consumption of *L. rhamnosus* GG had not led to a corresponding increase in *Lactobacillus* bacteremia.

XII. COMPARING PROBIOTICS

Many bacteria are tested to find a putative probiotic, yielding conflicting data, sometimes for the same organism. Comparisons between studies and organisms cannot be readily made because of nonstandardized dosing procedures, particularly for the number of bacteria and the duration of dosing. Pharmacokinetics, pharmacodynamics, safety, and the risk of acquisition of antimicrobial resistance have usually not been evaluated.[39]

Probiotic effects are strain-specific, illustrating the need to characterize the relationship between the dose, its duration, and effect on a strain-by-strain basis. When considering the pharmacokinetics of the probiotic organism, we want to know if the bacterial strain modifies intestinal flora. In determining the dose-response relationship, if there is failure to elicit an effect, is it because the organisms failed to reach effective levels at the site, or is it due to rapid elimination of the bacteria, or nonpersistence, or destruction?

It is unclear whether proposed consumption of a probiotic is to be on a regular daily basis throughout life, or irregular and dependent on symptoms. Borriello et al.[9] were unable to find published medical literature regarding the consumption of viable probiotics by hospital patients, some of whom may be predisposed to infection by probiotic bacteria.

Information is not readily available on (a) the equivalence or comparability of formulations in different preparations; (b) the distinction between spore or vegetative forms, powders, granules, tablets, liquids, and yogurts; or (c) adult and pediatric products. Intake data are not generally available for countries where such products are used. Nutritional studies may be needed in addition to toxicological studies, depending on (a) the nature of the product; (b) its intended use; (c) its anticipated intake; and (d) the impact of dietary intake on the spectrum of colonic flora, their metabolic functions, and bioavailability of nutrients.[40]

XIII. EVOLUTION OF GUIDELINES FOR PROBIOTIC SAFETY

Before new probiotic microorganisms and novel probiotic products are introduced into the market, their safety will need to be assured. There is vigorous debate on what constitutes appropriate safety testing for novel probiotic strains proposed for human consumption. Conventional toxicology and safety evaluation is of limited value in assessing the safety of probiotic bacteria.

In 1996 the European Union initiated the program Demonstration of Nutritional Functionality of Probiotic Foods (PROBDEMO CT96-1028), the aim of which was to provide and verify scientific evidence of claims for probiotic products. It established a list of safety criteria for probiotic foods (Table 1).

The European Commission's 5th Framework Program is addressing this complex subject in its collaborative project, Biosafety Evaluation of Probiotic Lactic Acid Bacteria Used for Human Consumption, due for completion in 2004. The project, Project 7 of the PROEUHEALTH cluster, is examining issues that include:

The presence and horizontal transfer of antibiotic-resistance genes
The detection of virulence factors
The evaluation of adverse immune effects
Probiotic survival, colonization and genetic stability in the human gut.

Project 7 is intended to formulate standardized premarketing safety testing and postmarketing surveillance of probiotics.

Table 1 PROBDEMO Criteria for Safety of Probiotics

1. The producer of food has the ultimate responsibility for supplying a safe food. Probiotic foods should be as safe as other foods.
2. If a probiotic food is a novel food, it is subject to legal approval according to the EU directive for novel foods.
3. When a strain has a long history of safe use, it will be safe as a probiotic strain and will not result in a novel food.
4. The best test for food safety is a well-documented history of safe human consumption. When a strain belongs to a species for which no pathogenic strains are known and for which other strains have been described that have a long history of safe use, it is likely to be safe as a probiotic food and will not result in a novel food.
5. When a strain belongs to a species for which no pathogenic strains are known but which do not have a history of safe use, it may be safe as a probiotic food but will result in a novel food and should be treated as such.
6. When a new strain belongs to a species for which strains are known that are pathogenic, it will result in a novel food.
7. State-of-the-art taxonomy is required to describe a probiotic strain, including DNA–DNA hybridization and rRNA sequence determination. This reasoning specifically applies to mutants of a probiotic strain.
8. In line with recommendation (1), strains that carry transferable antibiotic resistance genes (genes encoding proteins that inactivate antibiotics) should not be marketed.
9. Strains that have not been properly taxonomically described using the approaches indicated in (7) should not be marketed.
10. Strains should be deposited in an internationally recognized culture collection.

Source: Adapted from Salminen et al.[8]

FAO/WHO has convened a joint Working Group to draft guidelines for evaluating probiotics used in food.[41] The Working Group proposed a framework of strain identification and functional characterization, followed by safety assessment and Phase 1, 2, and 3 human trials. It recommended that probiotic foods be properly labeled with the strain designation, minimum numbers of viable bacteria at the end of shelf life, storage conditions, and manufacturer's contact details. The minimum tests required for characterization of safety are shown in Table 2. The Working Group further posited that assessment of lack of infectivity by a probiotic strain in immunocompromised animals would increase confidence in the safety of the probiotic.

The European Commission[42] is currently exploring a scheme with some similarity to the GRAS system in the United States to formulate a consistent approval procedure for the use of microorganisms in feeds and foods. Microorganisms associated with animal feeds are strictly regulated in Europe, but there is no formal mechanism for granting safety status to microorganisms in human food. This leads to inconsistencies where an organism or closely related strain with a long history of safe use in human foods is subject to strict safety assessment as an animal feed additive.

The scheme proposed is based on the concept of qualified presumption of safety (QPS), defined as "an assumption based on reasonable evidence" and qualified to allow certain restrictions to apply. The intention of the scheme is to have consistent generic safety assessment of microorganisms through the food chain without compromising safety standards. Case-by-case evaluations would be limited to aspects particular to the organism, obvious examples being acquired antibiotic-resistance determinants in lactic acid bacteria or toxin production in species known to contain toxigenic strains.

Qualifications to QPS approval could be (a) general, e.g., live or dead bacteria consumed directly by humans should be free of acquired resistance to antibiotics of importance in human or veterinary medicine, or (b) specific to an organism, e.g., bacteria from taxonomic groups containing toxigenic strains should be demonstrated free of toxigenic potential.

Broadly, the characteristics to be evaluated for QPS approval are:

Unambiguous identification at the claimed taxonomic level.
Relationship of taxonomic identity to existing or historic nomenclature.
Degree of familiarity with organism based on weight of evidence.
Potential for pathogenicity to humans and animals.
End use of the microorganism—Is it to be directly consumed? A component of a food product not intended to enter the food chain, but which may

Table 2 Joint FAO/WHO Working Group Recommendations to Evaluate Probiotic Safety

1. Determination of antibiotic resistance patterns
2. Assessment of metabolic activities (e.g., D-lactate production, bile salt deconjugation)
3. Assessment of side effects during human studies.
4. Postmarket epidemiological surveillance of adverse incidents in consumers.
5. If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production.
6. If the strain under evaluation belongs to a species with a known hemolytic potential, determination of hemolytic activity is required.

Source: Ref.[41]

adventitiously; or used as a production strain in a product intended to be free of live organisms? The end use of the microrganism will influence any qualifications imposed.

QPS status would not apply to a microorganism that commonly causes pathogenicity. If pathogenicity was limited to selected strains and its mechanism was testable, the microorganism might remain eligible for QPS status. The microorganism would not necessarily be considered a potential pathogen if there are infrequent reports of clinical isolates from severely ill people.

An example of how the process could proceed is summarized for *B. subtilis.* Enough is known to establish the identity of strains in the *B. subtilis* group. Its biology and pathogenicity are understood well enough to exclude problem strains. Some strains may qualify for QPS status, provisional to meeting the qualifications that (a) PCR-based evidence shows an absence of toxigenic potential; (b) production strains with toxigenic potential fail to produce detectable toxin levels in the production system employed; (c) the strain is free of acquired resistance to antibiotics of significance to human and veterinary medicine; and (d) it cannot produce antibiotics with structural similarities to those in human or veterinary medicine likely to encourage development of resistance.

XIV. SUMMARY

Probiotic bacteria should be unequivocally identified and defined with correct taxonomy.

Probiotic strains should be deposited in a recognized international culture collection for access by manufacturers, scientists, and regulators to ensure that organisms can be monitored for genetic drift and comparison with clinical isolates.

Novel probiotic strains from species with pathogenic, toxigenic or other adverse properties should be evaluated with scientific rigor.

Probiotic organisms should be systematically screened for antibiotic resistance and its transference.

Immunomodulatory effects of probiotics should be assessed in defined target populations.

Clinical studies should comply with the gold standard of randomized, double-blind, placebo-controlled design.

Probiotics in animal feed additives or veterinary products should be evaluated for their safety in the human food chain.

Labeling of probiotic products should accurately reflect content, shelf life, claimed attributes, and dose.

Following the introduction of novel probiotics, intake data should be gathered, especially for long-term consumption.

After market release of a novel probiotic, epidemiological surveillance for any associated adverse effects, particularly infection, should be instituted.

Clinical isolates should be compared with endogenous and probiotic strains to confirm their safety.

National reference centers should identify species and strain in clinical cases.

National clinical and epidemiological databases should include identity of organism, status of patient's underlying conditions, coexisting infections, and outcomes.

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Lactic Acid Bacteria as Animal Probiotics

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I. INTRODUCTION

The composition and metabolism of the gastrointestinal microbiota affects the performance of farm animals in many ways, especially young ones subjected to environmental stress. The indigenous microbiota that is established after birth interacts with the digestive and immune systems of the body, and its activities can be both beneficial and harmful to the host. The colonization of the different compartments of the gut by specific commensal bacteria, partly by means of association with the mucus layer or adhesion to the surface or epithelial cells, serves as a first defense barrier against invading microorganisms or toxic substances in the diet. In some species, especially in adult ruminants, the digestion of a fibrous diet is mainly based on the fermentative action of the bacteria in the rumen. In addition to digestive aid, the gut microbiota may produce substances or reprocess the refluxed host metabolites that are absorbed and utilized or excreted.[1,2]

In healthy animals, each part of the intestines is colonized by a typical microbiota, which is adapted to grow in a beneficial symbiosis with the host. Due to the intensive management methods of today, farm animals are very susceptible to enteric bacterial imbalance, leading to inefficient digestion and absorption of nutrients and retarded growth. To overcome these difficulties, diets have been supplemented with antibiotics,

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which have indeed proven very effective in decreasing diarrhea and promoting growth.[3–5] However, the development of resistant strains of harmful bacteria may interfere with the use of veterinary antibiotics[6,7] and decrease the efficiency of antibiotics per se. Possible residues in the animal products and cross-resistance with human pathogens might also result in health risks, which are so far not completely understood.[8,9] The possible transmission of antibiotic resistance from animals to humans together with increased awareness of consumer safety has led to the prohibition of antibiotics as feed additives for production animals and to restricting their use for therapeutic purposes only. In the European Union (EU), practically all use of feed antibiotics for production animals is prohibited, with the exception of coccidiostats for poultry. Further proposals to phase out the four remaining antibiotic feed additives still on the EU market (until January 2006) are in preparation.

For the above reasons there is wide interest in replacing feed antibiotics with more natural feed additives—probiotics. This term was used by Lilly and Stillwell[10] to mean a substance secreted by one microorganism that stimulated the growth of another. Parker[11] defined probiotics as organisms or substances contributing to optimal intestinal microbial balance. Fuller[12] interpreted "organisms or substances" as meaning live microbial feed supplements, excluding the possibility that the "substances" could be antibiotics. Synthesizing the above expressions, the authors consider animal probiotics to be live microorganisms that decrease the number of intestinal infections and/or increase production and/or improve food hygiene by contributing to a better gastrointestinal environment.

Since the early studies of Metchnikoff[13,14] of the favorable effects of soured milk products in humans, the most beneficial part of the intestinal microbiota has been suggested to be lactic acid bacteria (LAB). These organisms are most often found in commercial probiotic preparations,[15,16] but spore-forming bacilli or bifidobacteria may be used as well. The purpose of this overview is to introduce the composition and activities of the gut microbiota in farm animals and to condense the latest knowledge of LAB as potential performance enhancers. The term LAB includes members of the genera *Lactobacillus* and *Enterococcus* (formely *Streptococcus*) species *faecium* and *faecalis.* The following discussion will be concentrated on pigs and young cattle, which are developing ruminant behavior. Studies of other species, such as poultry, will be referred to only when necessary to help understand the probiotic concept. For non-LAB probiotics (bacilli, yeasts, etc.) and other animal species, the reader is referred to the reviews of Tournut,[17] Kozasa,[18] Vanbelle et al.,[19] and Huber.[20]

II. GENERAL ASPECTS OF THE GUT MICROBIOTA IN PIGS AND CALVES

A. Composition

The composition of the gut microbiota is known to vary due to many host-specific and environmental factors. Age and the gut site or the diet of the animal may be the most important examples of the former or the latter, respectively. Microbial communities in a certain part of the gut can be found in the lumen (attached to the feed particles or existing freely in the fluid), in association with the mucous epithelium, or in the bottom of the crypts.[2] A detailed discussion of the colonization factors believed to affect the gut microbiota has been given by Savage[21] and Tannock,[22] among others.

1. Pigs

The gastrointestinal (GI) tract of pigs is first inoculated with bacteria occurring in the reproductive tract of the dam and then by those existing in the immediate environment.[23] The stomach of the neonatal pig has been shown to be colonized by lactobacilli, streptococci, enterococci, and coliforms within 48 hours after birth, and strictly anaerobic organisms, such as bacteroides, can also be detected in the feces when the pig is a few days old.[24] In the suckling period, bacteria that can utilize the components of milk predominate in the upper tract,[25,26] and the milk constituents evidently largely determine which microbes can be implanted in the intestines. After the piglets start to consume creep feed and are finally weaned, an adult type of microbiota begins to develop in the upper (stomach and anterior small intestine) and lower (ileum, cecum, and colon) tract. At the same time the main site of bacterial fermentation changes from the stomach to the large intestine. In fact, the colonic microbiota of adult pigs resembles that of the rumen except for the lack of protozoa.[27,28]

The adhering LAB on the nonsecretory squamous epithelium of the pigs stomach are believed to serve as a source of inoculum for the lumen. However, this could not be confirmed by Henriksson and coworkers,[29] who studied the effect of removal of the *pars oesophagus* area. *Lactobacillus fermentum* and *Streptococcus salivarius* are the predominant strains in this area,[25,26] reaching the level of 10^8 CFU cm^{-2}. A similar layer of bacteria can be detected in the crop of chickens and in the stomach of humans and rats, although the adherent strains differ between the species. Recent data reveal that the LAB population in the pig's stomach may evolve during its life.[30,31] This means that the strains isolated from the young pig before weaning may not colonize the stomach of adult pigs, or vice versa.

Microbes other than LAB (*E. coli*, yeasts) often found in the stomach of pigs might be considered as transient, nonindigenous organisms, since they evidently cannot colonize the squamous area.[1] According to Blomberg and Conway,[32] the increased *E. coli* (K 88) growth in the anterior porcine gut is connected to changes in the LAB population of the squamous area.

The microbiota of the small intestine (SI) is affected by the bile salts and fast passage rate, but the same microbial groups as are found in the stomach can be cultured. The number of bacteria increases posteriorly due to slower flow rate and possibly lowered concentration of deconjugated bile acids. Jonsson[33] noted that the pig SI microbiota may be transient because evidence for adhesion is lacking. Muralidhara et al.[34] found coliforms and lactobacilli up to 10^7 and 10^8 CFU/g luminal contents, the figures being lower for the mucosal homogenates (10^5 CFU/g). However, Wadström et al.[35] demonstrated the in vitro adhesion of several strains of lactobacilli and streptococci isolated from the SI wall homogenates to the SI epithelial cells. Fuller et al.[36] reported the adhesion of *Streptococcus faecium* on the duodenal epithelium of chickens; the amount of attached bacteria was markedly higher in the macerated tissue homogenate than in the luminal contents.

Due to slow passage, the densest microbial population in pigs is found in the large intestine, the total number being 10^{10}–10^{11} CFU/g wet contents. *Bacteroides*, lactobacilli, and bifidobacteria are the most numerous, but enterococci and coliforms can also be found in high numbers.[33] The colonic microbiota has been mainly studied by sampling feces, but many workers have argued against this method (see Ref.[1]) as the fecal microbiota has been found to be different from the mucosal and luminal microbiota.[37]

2. Calves

At an early age the rumen of a calf has good physiological resources for the development of a microbiota. The inocula of the contents in the forestomachs is a natural event, obtained from the air, mother, etc.[38]

Cellulolytic and methanogenic bacteria can be found at the age of 3 days in the reticulorumen of the calf.[39] At the age of 1–3 weeks cellulolytic and lactate-fermenting bacteria and coliforms are present in the microbiota.[40,41] Lactate-fermenting bacteria are decreased after this period, and at the age of 9–13 weeks the ruminal microbiota of the calf is similar to that of an adult ruminant.

Lengemann and Allen[42] found that in milk-fed calves the development of the cellulolytic bacteria or the microbiota generally was slower than in calves fed with dry feeds. Nieto et al.[43] suggested that weaning of the calf from milk to dry feeds caused a more rapid appearance of protozoa in the rumen. Protozoa could be found at the age of 8 days. Moreover, artificial inoculation did not affect the establishment of the culturable bacteria, but after inoculation with rumen contents, protozoa (*Entodinia, Diplodinia*, and *Holotrichs*) appeared at the age of 6 weeks.[38] Normally ciliate protozoa were not found in the rumen earlier than the age of 13 weeks.[40]

After birth, milk or liquid milk replacers are the main feeds for a nonruminant. Liquid feed also passes the reticulorumen via esophageal groove to the abomasum and further into the small intestine. Therefore it is natural that one of the first groups of microorganisms in the rumen is LAB and that rumen microbiota has no great effect on feed digestion at an early age. This means that in the nonruminant calf disorders of the digestive tract may be treated as with piglets. However, Marounek et al.[44] suggested that metabolic products of some rumen microorganisms might have probiotic-type effects in the calf even at an early age.

The small and large intestinal microbiota of calves resembles that of the rumen (reviewed by Jonsson[33]) and is also affected by diet and age. Marshall et al.[45] isolated adherent lactobacilli from the epithelium of the esophageal groove, omasum, abomasum, and duodenum at levels of 10^4–10^7 cm^{-2}. According to Gilliland et al.[46] the numbers of lactobacilli and coliforms in the small and large intestine vary between 10^6–10^7 and 10^8–10^9 and 10^5–10^7 and 10^8–10^9/g dry weight of the gut contents, respectively.

B. Digestion

1. Carbohydrates

Cranwell et al.[47] showed that in the stomach of piglets, large amounts of lactic acid are produced by LAB, mainly from lactose and glucose (Table 1). This may be essential for pH regulation and formation of an acid barrier, since HCl production is still limited in the suckling period. In a later study,[48] heavy lactate production was observed to inhibit HCl secretion, indicating that the regulation is based on hydrogen ion concentration and not on the acid per se. Lactate constitutes 80–90% of the total organic acids in the stomach (50–80 mmol/L) of a suckling pig,[49] the proportion being much lower (50%) in older animals on a creep diet.[50,51] Sugars are also the most likely substrates for small intestinal bacteria, but the real quantity of the fermentation may be limited. However, organic acids up to 50–100 mmol/L can be found, lactate predominating and acetate accounting for most of the VFA. Part of the organic acids in the small gut may be contributed by the digesta emptying from the stomach.[23]

Table 1 Contribution of Microbes to Digestion of the Host in Different Compartments of the Gut

	End products of bacterial digestion		
Substrate	Stomach	Small intestine	Cecum + colon
Carbohydrates	Lactic acid	Lactic acid; acetate	Volatile fatty acids
Protein and N compounds	Ammonia Amines (amino acids)	Ammonia Amines (amino acids)	Ammonia Amines
Lipids	Fatty acids	Fatty acids; deconjucated bile, modified cholesterol	De novo synthesis of fat; hydrogenated fatty acids; modified cholesterol

The large intestine allows efficient bacterial fermentation to take place, especially in adult pigs. Degradation of plant cell-wall carbohydrates, mucin, and other endogenous secretions results in organic acids in a series of reactions, as in the rumen.[52] The amount of organic acids in the chyme varies between 150 and 200 mmol/L,[50,51] with acetate, propionate, and butyrate accounting 60, 30, and 15 mol%, respectively, and lactate occurring only occasionally in trace amounts. VFA absorbed from the large gut can represent 15% of the total net energy requirement of the growing swine.[53]

2. Protein and Nitrogen Compounds

Bacteria unlikely possess any significant proteolytic activity, especially in the upper tract, but amino acids, peptides and urea are used as N sources (Table 1). Almost all amino acids can be deaminated or decarboxylated to yield ammonia and amines.[54] Hill et al.[55,56] reported that *E. coli* is the main amine producer and that feeding LAB significantly reduced amine formation in young pigs. In general, ammonia and amine formation is seen as a harmful process of the gut microbes, and the prophylactic effect of antibiotics is believed to be based on lower production of these N compounds.[4]

Urea is formed in the N turnover of tissues and is secreted into all parts of the GI tract in digestive juices or straight across the luminal wall.[57] It is likely that the microbes in close association with the epithelium degrade urea and liberate ammonia in the lumen. Ammonia may be incorporated into bacterial N or absorbed and converted back to urea by the liver for urinal excretion or recycling into the gut. This enterohepatic circulation of urea[58] demands energy from the host and may irritate the gut mucosa. However, in the cases of low N intake, this process might conserve nitrogen for the host.[23]

3. Lipids

The intestinal microbiota contributes to lipid metabolism of the host in two different ways: first, bacteria can digest dietary and endogenous lipids by lipases and hydrogenate the free fatty acids, and second, they can deconjugate bile acids and modify cholesterol metabolism (Table 1). The apparent digestibility of fats may be decreased by the activity of bacteria, since hydrogenated fatty acids are less absorbable than unsaturated ones, but evidently also due to the de novo fat synthesis in the large gut.

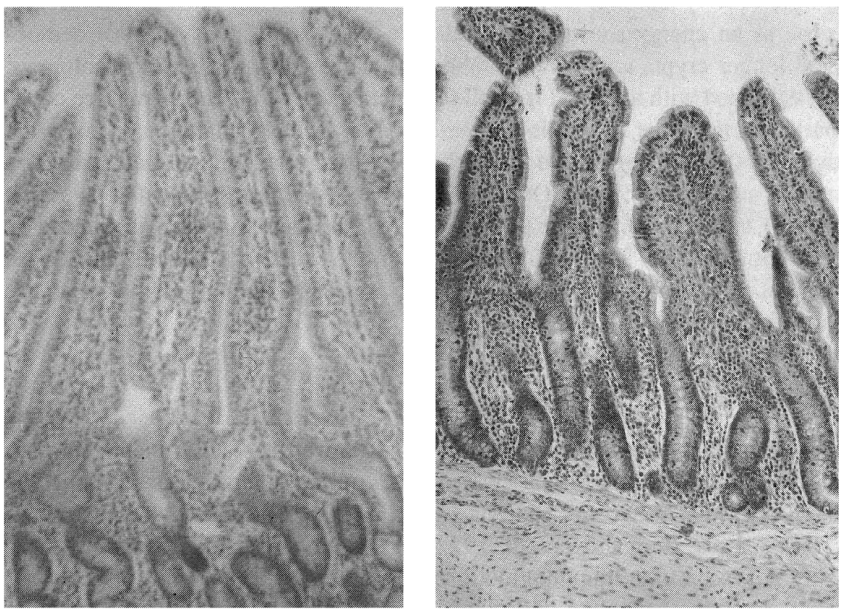
Free bile acids are conjugated with taurine, glycine, sulfate, or glucuronide. The primary bile acids are deconjugated by the gut microbiota, among others by lactobacilli and bifidobacteria,[59] to less soluble and less absorbable secondary products.[60]

The secondary bile acids are partly converted to tertiary bile products by the gut microbiota and hepatic enzymes, and they may be toxic to the host.

The gut microbiota is also involved in the cholesterol metabolism, because bile acids are synthesized from cholesterol, although the mechanism has not thoroughly been studied in pigs or calves. Evidence from the other species suggests that the gut microbiota decreases the body pool of cholesterol by catabolizing and making it less absorbable.[61] Many attempts have been made to reduce serum cholesterol, considered an additional risk factor for coronary heart disease by intake of LAB products. Some trials have been successful, but response has been lacking in others.[62]

C. Gut Wall Function

The gut wall in all parts of the intestine is organized in a special way. The folded mucosa is clothed by finger-like projections, villi, which are in turn clothed by absorbing enterocytes or mucus-secreting goblet cells.[63] Between the villi exist the crypts of Lieberkühn, where epithelial cells proliferate, extending down to the lamina propria (Fig. 1). During migration from the bottom of the crypts to the villus tips, the mucosal cells differentiate and mature, during which process the amount of digestive enzymes increases.[64] The results obtained from studies comparing germ-free and conventional animals show a marked interaction between microbiota and the structure of the intestinal mucosa.[65,66] In general, germ-free animals possess longer villi, shallower crypts, and, as a result of these morphological differences, higher enzyme activity than their conventional counterparts. According to the suggested mechanism, the gut bacteria per se or their metabolites increase the rate of mucosal cell renewal.[67] According to Visek,[68,69] the products of



(a) (b)

Figure 1 Photographed jejunal and ileal mucosa of a weaned pig describing mucosal architecture. Tissue slices stained for light microscopy (×16) with hematoxylin and eosin: (a) jejunum, (b) ileum.

bacterial nitrogen-metabolism, ammonia and amines, have a harmful influence on the mucosal structure. Hampson,[70,71] Miller et al.[72] and Cera et al.[73] have demonstrated in piglets a sharp decrease in villus length and an increase in crypt depth after weaning, and evidently the radical change of the gut microbiota connected to weaning contributes to this phenomenon.

Sakata[74] demonstrated in rats that intraluminally infused VFA accelerate the crypt cell production rate and increase gut wall mass. The stimulation was most efficient with butyrate. The effect may be systemic rather than local, since cecally administered VFA enhanced crypt cell production rate in jejunal samples. Roediger[75] reported that in rats and humans butyrate is even preferred to glucose as an energy source for colonocytes. Hill and Cowley[76] demonstrated longer crypts and lower numbers of mature goblet cells in the colons of mice equipped with a normal microbiota in comparison to their germ-free counterparts. On the other hand, dietary antibacterials, which obviously decrease the activity of the gut microbiota, have been shown to decrease the gut wall mass and stimulate nutrient absorption.[68,5] Yen et al.[77] among others, noted in young pigs that gut wall mass is reduced by dietary antibacterials. They later speculated[78] that this might lead to lower fasting energy consumption because the intestinal epithelium is one of the most active tissues in the body.

D. Gut Immune System

Gut-associated lymphatic tissue (GALT), existing immediately under the outer cell layers of the mucosa, forms the first host-specific defense barrier against the antigen exposure of harmful bacteria and other antigens in the diet (for reviews, see, e.g.[79–81]). The secretory immunoglobulins of GALT (mainly sIgA, with sIgG also present) are complexed with the goblet cell mucine and are the main specific protecting mechanism. The lymphocytes that secrete IgA arise in the Peyer's patch lymphoid regions. The secretory IgA is in a dimeric form, possessing a specialized peptide j-chain, which binds the heavy chains of the immunoglobulin. It is believed that the IgA system is activated by the local antigens near the mucosal surfaces.[82] The epithelial cells synthesize a receptor for this peptide known as a secretory component, facilitating the binding of IgA to the mucin and distribution over the external mucosa as a protective layer. Macrophages and cytotoxic T cells are responsible for the cell-mediated immune reactions of the gut. It is obvious that the gut microbiota and GALT interact together in an important manner, since antibodies regulate the colonization of microbes on the epithelium. On the other hand, certain indigenous gut bacteria can exist in close association with this mucin-antibody "painting," evidently contributing to the defense effect. Moreover, the normal microbiota of the gut is believed to stimulate the immune defense of the GALT. It is not known how the GALT system distinguishes between indigenous bacteria and harmful pathogenic bacteria, which do not belong to the normal habitat of the gut. Savage[21] speculated that indigenous microbiota may have common antigens with the host.

III. THE POTENTIAL AND POSSIBLE MODES OF ACTION OF LAB AS BIOLOGICAL PERFORMANCE ENHANCERS–PROBIOTICS

A. Competitive Exclusion

The pioneering evidence of the competitive exclusion concept was obtained from chickens by Nurmi and Rantala.[83] Newly hatched birds do not obtain the normal gut microbiota of the adult, due to modern management methods. Since a normal microbiota is lacking, the

intestines of the birds are easily colonized by pathogens, most often by salmonellae or coliforms. It is rare that the infected broilers get sick due to *Salmonella*, or even show decreased growth, but as opportunistic organisms *Salmonella* might contaminate poultry food products. When chickens were inoculated just after birth by the fecal contents of an adult bird, the frequency of *Salmonella* infections was radically reduced and the number of *Salmonella* needed to colonize the ceca of the birds increased.

Much work has been directed to describe the exact components of competitive exclusion (for reviews see Refs.[84–86]). According to Impey and Mead[87] the main factors are competition for the receptor site on the gut wall, production of VFA and/or other antibacterial substances by the anaerobic microbiota, and competition among different bacteria for limiting nutrients. The specific role of LAB as a probiotic for live poultry was extensively discussed by Juven et al.[88] Competitive exclusion has not been studied in pigs or calves as such, partly due to the management differences between these animal species, but treatment with selected gut bacteria, mainly LAB, has been examined in detail during the past decades. Fuller[12] listed the possible modes of action of such selected probiotics as follows: (a) suppression of viable counts of pathogens and harmful bacteria, (b) alteration of microbial metabolism (enzyme activity), and (c) stimulation of the immune response (see also Table 2).

1. Antagonism: Production of Organic Acids or Specific Antibacterials

LAB produce many kinds of metabolites, which might affect the other microbes in the gut. Lactic acid produced by both homolactic and heterolactic strains reduces the pH in the luminal contents, which is most obvious in the stomach of neonatal piglets.[89,47] Moreover, acetic acid and H_2O_2 excreted by heterolactic strains may be

Table 2 Proposed Mechanisms of Beneficial and Detrimental Effects of LAB Probiotics

Response	Proposed mechanisms	Main site of action
Beneficial		
Suppression of harmful bacteria	(1) Production of antibacterial compounds	S,SI
	(2) Competition for nutrients	S,SI,LI
	(3) Competition for colonization sites	S,SI
Microbial/host metabolism	(1) Production of enzymes that support digestion (e.g., lactase)	S,SI
	(2) Decreased production of ammonia, amines or toxic enzymes	SI,LI
	(3) Improved gut-wall function	
Improved immune response of host	(1) Increased antibody levels	SI,(LI)
	(2) Increased macrophage activity	SI,(LI)
Detrimental		
Competition for nutrients with host	(1) Consumption of glucose	S,SI
	(2) Consumption of amino acids	S,SI

S = stomach; SI = small intestine; LI = large intestine.
Source: Adapted from Refs.[12,87]

toxic to some other bacteria.[90] It is well documented that organic acids and H_2O_2 produced by LAB are inhibitory against coliforms, salmonellae, and clostridia in vitro, but convincing in vivo evidence is still lacking.

Several high molecular antibacterials, such as acidophilin, acidolin or reuterin and nicin, have been described as being produced by lactobacilli and streptococci in vitro, respectively.[88,91] However, there is limited evidence that such substances can really be active in the intestines, and many researchers believe that the inhibitory effects are accounted for by the lower pH, organic acids, or hydrogen peroxide. Klaenhammer[92] suggested that the significance of *Lactobacillus* bacteriocins against undesirable intestinal organisms is questionable because of the narrow range of activity of these compounds.

Although the mechanisms of antagonistic properties of LAB are somewhat uncertain, there is some evidence that such a phenomenon takes place in the gut (Table 3). Muralidhara et al.[34] treated piglets immediately after birth with a human isolate of *Lactobacillus lactis.* A clear coliform-suppressing response in fecal samples was noted, but the number of lactobacilli was not affected. Moreover, after the treatment was stopped, a continued reduction of coliform numbers was observed. Ratcliffe et al.[93] fed piglets from 2 days of age with *L. delbrueckii* ssp. *bulgaricus* or *L. reuteri* fermented milk or nonfermented control milk. Both types of fermented milks decreased the numbers of coliforms and pH throughout the intestines. Since lactic acid added to the control milk gave similar results, the authors concluded that the favorable effects of fermented diets were due to the lower pH produced by lactic acid. Underdahl[94] inoculated gnotobiotic pigs with three virulent strains of *E. coli*, all of which developed severe diarrhea. Treatment with *Enterococcus faecium* reduced the severity of diarrhea, and treated pigs recovered earlier and gained weight normally compared to their untreated littermates. The better performance of treated pigs was associated with a lower number of both organisms in the small intestine and cecum. Ozava et al.[95] treated piglets and calves reared on an antibiotic-containing diet with *E. faecalis* and noted increased numbers of lactobacilli, streptococci, and bifidobacteria in the feces of experimental animals. In addition, yeasts and salmonellae were suppressed due to the treatment.

Barrow et al.[26] showed decreased counts of *E. coli* in the stomach of piglets fed a combination of *S. salivarius* and *L. fermentum.* Treatment with *L. delbrueckii* ssp. *bulgaricus* in pigs showed that the organism produced a substance that seemed to neutralize the effect of enterotoxin released from coliforms.[96] Additional evidence of the

Table 3 Data Supporting the Antagonistic Properties of LAB Against Harmful Gut Bacteria

Target host	Organism	Response	Refs.
Small piglets	*L. lactis*	Decreased *E. coli* in feces	[34]
Small piglets	*L. reuteri* *L. bulgaricus*	Decreased pH and *E. coli* in the gut	[93]
Gnotobiotic pigs, *E. coli* challenged	*S. faecium*	Decreased *E. coli* Less scours	[94]
Small piglets	*S. faecalis*	Increased LAB and bifidobacteria Suppressed salmonella, and yeasts	[95]
Pigs	*L. fermentum* *S. salivarius*	Decreased *E. coli* in stomach	[26]
Pigs, calves	*L. bulgaricus*	Neutralized *E. coli* toxin	[96,97]
Calves	*L. acidophilus*	Suppressed *E. coli*	[46]

antienterotoxic property of LAB was obtained from trials with calves by Schwab et al.[97] Gilliland et al.[46] observed lower numbers of coliforms in the ileum of calves fed with host-specific *L. acidophilus.*

In spite of many observations supporting the antagonistic properties of LAB, negative results also occur. Pollmann et al.[98,99] did not observe any effect on the fecal microbiota of *L. acidophilus*–treated piglets. It is noteworthy, however, that the fecal sample is probably not a valid indicator of the intestinal ecosystem.

2. Adhesion

Adhesion or close association of LAB probiotics to the epithelial cells may further contribute to competitive exclusion. First, LAB that grow relatively slowly but attach to the gut wall can transiently colonize and inoculate the luminal contents. This seems to be obvious, for example, in the stomach of pigs[26] and in the crop and cecum of chickens.[100,101] Second, if LAB occupy the adhesion receptors on the surface, the harmful bacteria relying on them may be eliminated from the gut (Fig. 2). This is, of course, a valid principle only if pathogens and LAB have parallel attachment mechanisms. Davidson and Hirsch[102] blocked the colonization of pathogenic *E. coli* K88 with a nonpathogenic *E. coli* strain. Similarly, *Lactobacillus* cells or cell wall fragments were reported to prevent adhesion of *E. coli* on human uroepithelial cells.[103,104]

The association mechanisms of intestinal bacteria in general and of LAB on the gut surfaces have been discussed by Savage.[105] Gram-negative bacteria, e.g., pathogenic *E. coli*, attach to the target cells via proteinaceous projections (fimbriae), but lactobacilli seem to adhere to the gut wall with extracellular substances containing polysaccharides,

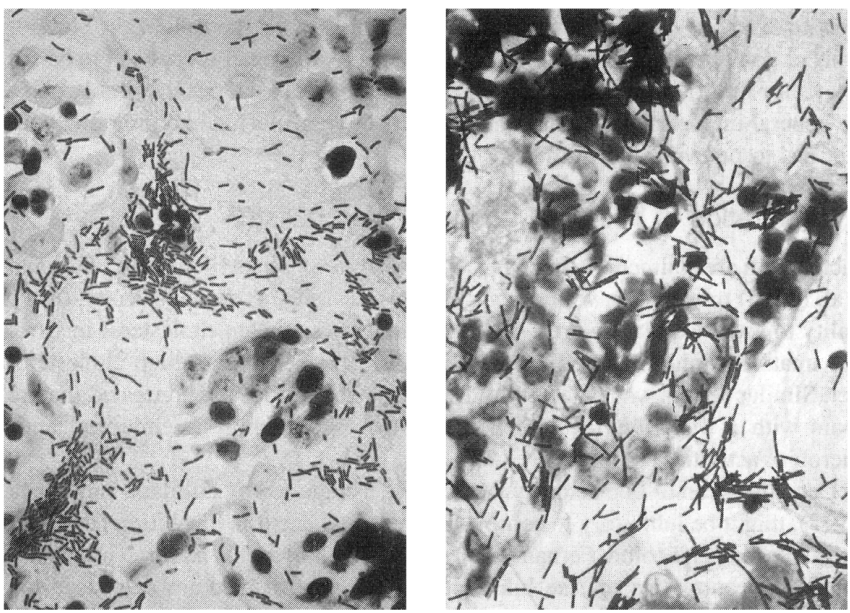


Figure 2 Two host-specific *Lactobacillus* strains differing in their ability to adhere to the epithelial cells of the small intestine of a pig: (a) adherent, (b) nonadherent.

proteins, lipids, and lipoteichoic acids. The role of the latter was discussed in detail by Tannock.[106] Lipoteichoic acids are glycerolphosphate polymers of the cell wall of lactobacilli, covalently linked with glycolipids, containing both hydrophilic and hydrophobic regions. Sherman and Savage[107] detected macromolecular protein complexes rich in lipoteichoic acids in *Lactobacillus* strains, some of them known to associate with epithelial surfaces. Appearance of acidic carbohydrate-rich material between attached bacteria and epithelium was also supported by the findings of Brooker and Fuller[108] with electron microscopy. Lipoteichoic acids may also participate in attachment of streptococci to mammalian cells.[106]

Wadström et al.[35] observed a number of *Lactobacillus* strains from the small intestine of pigs containing carbohydrate capsule polymers and possessing high hydrophobicity. Heat and protease treatment impaired these surface functions. They considered capsule formation to be the most important determinant of the intestinal colonization of lactobacilli in pigs. Henriksson et al.[109] reported that the adhesive determinants of *Lactobacillus fermentum* on the porcine gastric squamous epithelium are proteinaceous, although carbohydrates seemed also to be involved. Attachment ability of the rough and smooth variants of *L. fermentum* differed; the former was lacking the adhesion protein. However, it was hypothesized that during colonization the rough variant is needed to reach the epithelium and the environmental conditions induce the growth of smooth variant, which in turn binds efficiently to the mucosa.

Knowledge of the adhesion properties of LAB has been markedly increased during recent years, but many microbe-epithelium interactions other than those mentioned above certainly exist in the gut. The role of the components of goblet cell mucin (e.g., sialic acid) has been speculated to be the key factor in the mucosal association of the nonpathogenic intestinal bacteria. The general importance of adhesion for the competitive exclusion and probiotic concept needs further study.

B. Alteration of Microbial and Host Metabolism

Lactobacilli are claimed to affect the cholesterol metabolism of the host (Table 4). Gilliland et al.[110] treated pigs with a *Lactobacillus acidophilus* strain selected for its ability to grow well in the presence of bile and to assimilate cholesterol in vitro. The treatment inhibited the increase in serum cholesterol on a high-cholesterol diet. Similar results was obtained by Danielson et al.,[111] who treated mature boars with *L. acidophilus* screened in vitro for anticholesteremic and antimicrobial activities. Furthermore, treatment of laying hens with a mixture of LAB and fungi was shown to reduce the cholesterol level in eggs.[112]

Deconjugation of bile acids by lactobacilli, as reported by Gilliland and Speck,[113] might be inhibitory to some other intestinal bacteria inhabiting the lower small intestine and colon. Fernandes et al.[114] reported that addition of physiological concentrations of free bile acids to the growth medium decreased the growth and antimicrobial activity of *L. acidophilus.* Observations of Tannock et al.[115] revealed that bile salt hydrolase activity in the ileum of mice was reduced by 86% or 98% in the absence of lactobacilli or both lactobacilli and enterococci, respectively, compared to conventional animals.

Besides affecting cholesterol and bile acid metabolism, LAB are claimed to reduce the intestinal production of harmful nitrogen compounds. Pigs fed *L. acidophilus*–fermented milk showed less intestinal amine production than the pigs fed untreated control milk.[55] Also, the major site of amine production changed from the small intestine to the cecum in the treated pigs. When various gut bacteria were tested in vitro, E. coli was

Table 4 Data Supporting the Beneficial Shifts in Microbial or Host Metabolism by Feeding LAB Probiotics

Target host	Probiotic	Response	Refs.
Pigs	*L. acidophilus*	Decreased serum cholesterol	[110]
Calves	*L. acidophilus*	Formation of inhibitory bile acids	[113]
Pigs Humans	*L. acidophilus*	Decreased amine production	[55,117,118]
Humans	*L. acidophilus*	Decreased production of carcinogenic N compounds	[46,117]
Humans	*L. rhamnosus* + *Propionibacterium freudenreichii*	Reduced fecal mycotoxin level	[157]
Rats	*L. bulgaricus*	Hydrolytic enzymes which improve digestion	[119]
Chicks	*Lactobacillus* sp.	Increased amylolytic activity	[120]
Pigs	*Lactobacillus* sp.	Improved β-glucan hydrolysis	[121]
Pigs	*Lactobacillus* sp.	Increased activity of brush-border enzymes	[123]

noted to be the most efficient amine producer in pigs.[56] The carcinogenic fecal enzymes β-glucuronidase, nitroreductase, and azoreductase were reported to decrease in humans on a *L. acidophilus*–containing diet.[116,117] Intestinal production of free amines was noted to decrease in rats administered aromatic nitro- and azo-compounds as well as an amine-glucuronide compound.[118]

Ingested LAB produce and release hydrolytic enzymes, which might aid digestion in farm animals, particularly during the early life of calves and piglets. Humans suffering from β-galactosidase deficiency may digest lactose in yogurt better than the same amount of lactose in milk. Indeed, rats fed yogurt had increased β-galactosidase activity in their small intestines and the enzyme seemed to be of bacterial origin.[119]

Some observations suggest that lactobacilli could contribute to the digestion of more complex carbohydrates than lactose. Champ et al.[120] isolated three *Lactobacillus* strains from chicken crop, which showed amylolytic activity. The best amylolytic strain resembled *L. acidophilus*, producing maltose, maltotriose, and traces of glucose from amylopectin. Optimum pH and temperature of the amylase were 5.5 and 55°C, respectively. Jonsson and Hemmingsson[121] found β-glucan degrading lactobacilli up to 10^8 CFU/g from the feces of 3- and 35-day-old piglets fed a creep diet containing 2% β-D-glucan. Glucanolytic probiotics might be very useful in the diets of poultry and pigs containing barley and oats, because host enzymes evidently cannot degrade β-D-glucan and because it interferes with starch digestion.

Probiotics containing LAB might also affect the levels of the host brush border enzymes, as speculated by Parker.[122]. Collington et al.[123] fed piglets with antibiotics

or LAB probiotics and reported increased lactase and sucrase activities in the small intestinal mucosa with both treatments. It may be speculated that the effect of both treatments was not direct, but was due to lower production of harmful bacterial metabolites, which irritate the mucosa and affect the life span of the enterocytes. In contrast, Whitt and Savage[124] found no direct influence by several indigenous lactobacilli on the enzyme activities of the duodenal enterocytes in germ-free and ex–germ-free mice. This led the authors to conclude that any beneficial effects of probiotic bacteria on the function of gut mucosa may result from the interaction with the whole microbiota inhabiting the lumen (e.g., pathogens).

C. Stimulation of Immunity

An example of the interaction between microbes and the immune system is furnished by conventional animals equipped with a complete indigenous microbiota, which have higher immunoglobulin levels and phagocytic activity than their germ-free counterparts.[125] Therefore, it has been suggested by many authors on the basis of encouraging research results that probiotic bacteria could enhance immunity both locally on the mucosal surfaces and at the systemic level. *E. faecium* used as a monoassociate to germ-free mice reduced the counts of *Salmonella* (intravenous challenge) in the spleen, implying a systemic response.[126] In contrast, Kluber et al.[127] did not observe any responses in in vivo cell-mediated immunity in artificially reared piglets treated with *E. faecium. Per os* administration of *L. acidophilus* and *L. casei* increased phagocytic function of macrophages in mice.[128] Moreover, *L. acidophilus* and *S. thermophilus* activated macrophages and lymphocytes, whether given perorally or intraperitoneally.[129] Macrophage activation was also noted by Saito[130] with *L. casei* in mice. Lessard and Brisson[131] fed piglets rehydrated skim milk powder fermented with a mixture of lactobacilli and reported slightly increased serum IgG levels. A local immune enhancement with LAB was reported by Perdigon et al.[132] in mice. Oral intake of *L. casei* increased the IgA production secreted to the intestinal lumen, providing mucosal defense against *Salmonella typhimurium.* These examples suggest that LAB can modulate immunity. However, there is little evidence whether this kind of response can be obtained in commercial circumstances where animals harbor a complex microbiota in their intestines.

IV. SELECTION CRITERIA FOR LAB TO BE USED AS PROBIOTICS

Many potential improvements in animal performance may be achieved with LAB probiotics, as demonstrated by the research examples above, but the responses obtained in field trials may vary. Much of the discrepancy between basic concepts and real life is obviously accounted for by the characteristics of the strains used, and therefore anyone aiming to develop a good probiotic has to carefully evaluate the selection criteria.

LAB probiotics for animal use marketed so far are freeze-dried bacteria, often belonging to the genera *Lactobacillus* spp. or *Enterococcus* spp. Often the strains used are suitable intestinal bacteria or dairy starters, but products based on host specificity also exist. A number of criteria must be fulfilled by any successful probiotic. The main criteria used in the authors' laboratory are listed in Table 5 (see also Refs.[12,133]). First, a probiotic must be a nonpathogenic representative of the normal intestinal microbiota, most preferably host specific, and it must maintain its activity in the presence of high acidity in the stomach and high concentration of bile salts in the small intestine. Second,

Table 5 Criteria Used in the Authors' Laboratory for Screening Probiotics

Method	Basis
1. Acid tolerance	Survival during passage through the stomach and duodenum
2. Bile tolerance	Survival during passage through the upper small intestine
3. Acid production (from glucose and lactose)	Production of efficient "acid barrier" in the upper gut
4. Production of antimicrobial substances	Competition with pathogens
5. Adhesion of intestinal mucosa	Efficient colonization, exclusion of other microbes from adhesion sites
6. Heat tolerance	Survival during pelleting of creep feed
7. Tolerance of feed antimicrobials	Use possible with medicated feed
8. Good technological properties	Strain stability, growth on large scale, survival in product

a good probiotic must be able to grow and metabolize rapidly and exist in high numbers in the gut. Third, an ideal probiotic strain may colonize some part of the gastrointestinal tract transiently, for this adhesion to the mucosal surface is desirable. Fourth, it must produce organic acids efficiently and should have specific antimicrobial properties against harmful bacteria. Finally, it must be easy to produce, survive growth in a large-scale production system, retain its viability under storage and field conditions, and be cost-effective to use in farm animals.

There exists no one strain that completely fulfills all these criteria. Much variation in selection variables can be observed among isolated gut lactobacilli, as judged by our own experience (Table 6). In practice, the choice of an economically feasible probiotic is always a compromise between microbiological, production, and performance-promoting properties of the strains tested.

A. Acid and Bile Tolerance

High acidity in the stomach and high concentration of bile components in the proximal small intestine are the first host attributes that affect the strain selection. Conway

Table 6 Selection Characteristics of LAB Strains Isolated from Intestines or Feces of Pigs in the Authors' Laboratory

Strain type	*N*	Lactic acid production[a]	% L/D lactate	Heat tolerance[b]	% Bile[c] tolerant	% Acid[d] tolerant
L. acidophilus	15	0.39–1.33	50–100	60.4–69.5	40	30
L. fermentum	25	0.38–1.68	47–93	62.5–69.5	44	88
L. delbrueckii	4	0.60–1.20	47–63	64.0–65.4	—	—
Lactobacillus sp.[e]	13	1.03–2.07	39–70	65.5–69.0	39	92

[a]MRS broth, 1% glucose.
[b]Maximum temperature fully tolerated for 6 minutes.
[c]MRS broth, 0.3% Oxgall, no inhibition.
[d]MRS broth, pH 4.00, good growth.
[e]Adhesion to small intestinal cells of pig observed.[158]

et al.[134] incubated LAB strains in phosphate-buffered saline at pH 1, 3, and 5 for 0–4 hours at 37°C to screen human strains for their ability to survive in the stomach. Aspirated stomach juice obtained through a nasogastric tube after a 4-hour fast was used in this study. The strains detected showed variable survival with this method, and it was reported to be a valid tool to find potential microbes. Jonsson et al.[135] used fistulated pigs to test the in vivo survival of orally fed lactobacilli during transit through the upper tract. The cannulas were inserted distal to the pylorus and in the terminal ileum.

Gilliland et al.[136] observed great variability among *L. acidophilus* strains isolated from calf intestinal contents in their ability to grow in vitro in the presence of bile salts. When a strain exhibiting low tolerance to bile and another strain exhibiting high tolerance to bile were administered orally to calves, the more resistant strain caused a greater increase in numbers of facultative lactobacilli than the one possessing low tolerance.

B. Production of Antimicrobial Substances

The in vivo evidence for the production of specific bacteriocins by LAB is limited, and subsequently the spectra of these substances seem to be quite narrow. Moreover, due to methodological difficulties (e.g., pH elimination), the preferred method might be to screen the candidate strains for their ability to produce organic acids. Most commonly simple sugars, such as glucose or lactose, are used as carbon sources. Production of H_2O_2 can also be used, as was done, for example, by Jonsson and Olsson.[137]

C. Adhesion and Growth in the Gut

The problem of adhesion stems from the basic controversy as to whether strains are fed continuously or just once. In the latter case, good adhesion is a crucial property for the transient colonization of the probiotic. Additionally, attachment of probiotics to the gut wall may block the colonization of harmful bacteria on the mucosa. The adhesion test is usually made by incubating the strain and intestinal cell suspension together, and then verifying the binding by visual judgment with a microscope (see Fig. 2). Since visual judgment is not an objective method, radiolabeled cultures have been used by some authors. The test methods have been described by Fuller et al.[25] and Conway et al.[134] among others.

Although the existence of mucosally attached microbes has been experimentally proven, and thus adhesion provides a sound basis for the development of probiotics, much argument has been directed against its use. First, during harvesting of epithelial cells variable amounts of mucin are bound to the cells and thus interfere with the assay. In contrast, mucin may be essential for the gut wall association of some bacteria. Although it has been suggested that adhesion is host specific, recent findings put this in doubt.[138] The adhesive strains on the mucosa might vary according to the age and diet of the animal. Jonsson[139] did not observe permanent establishment of a host-specific *Lactobacillus* strain in pigs, although it adhered in vitro to the squamous epithelial cells. Finally, it may be posited that if the inoculation of the animal is not done immediately postpartum, the indigenous microbiota developed near the mucosa resists the attachment of the probiotic bacteria.

Because of the above options, it would seem that the best method of using probiotics is continuous inoculation. However, even with continuous feeding it is still important to screen the probiotic candidates according to their ability to survive and grow in the gut, and attachment ability is also a recommended feature. There exists limited knowledge

of the minimum effective dosage of probiotics when they are administered continuously. The fact that viable counts of probiotic organisms are found in feces is not proof of proliferation or metabolism during passage through the tract. Although it appears, according to the literature and our experimental data, that 10^6–10^7 CFU/g feed is necessary for a consistent effect, it may be reasonable to conclude that the effective dosage appears to be a strain-specific feature depending on survival and adhesion properties and the specific growth rate of the organism.

D. Feed Antimicrobial Resistance

Probiotics are often mentioned as natural substitutes for feed antibiotics, but in some cases it may be feasible to combine probiotic and antibiotic treatments to obtain an extra advantage. As stated above, the natural microbiota resists the invasion of both harmful and probiotic bacteria. If the natural microbiota is weakened by the use of a feed antimicrobial, the probiotic bacteria may be more easily established in the guts of target animals. There exist some preliminary results in the authors' laboratory that support this idea. On the other hand, by combined treatment the level of antibiotics needed could decrease.

According to Pollmann et al.[99] a *Lactobacillus* culture in combination with lincomycin may have an additive effect. Harper et al.[140] treated growing swine with a *Lactobacillus* probiotic or virginiamycin or both, but no interaction between treatments occurred. However, the viability of the cultures in the medicated feed was not monitored. Dutta and Devriese[141] investigated the minimal inhibitory concentrations of some commonly used feed antimicrobial agents against lactobacilli isolated from pigs, cattle, and poultry. The percentage of resistant strains of all isolates varied in pigs, cattle, and poultry between 2 and 70, 10 and 95, and 8 and 83, respectively, depending on the drug and suggesting a potential for the combined treatment of antibiotics and LAB probiotics.

E. Technological Properties

The production process of probiotics involves mass growth in fermentors, concentration, and subsequently, in most cases, freeze-drying steps. Probiotics may be used as high-activity (10^8–10^{10} CFU/g) dry preparations with a dosage of a few grams per animal per day, or they may be mixed at the rate of 10^6–10^7 CFU/g in the meal diet, often followed by pelletizing. In the latter case the probiotics must tolerate heat (60–80°C, 5–10 min) and extremely high physical pressure. As far as the authors' know, there exists no *Lactobacillus* strain that tolerates pelletizing in an economically feasible way. However, enterococci (e.g., *E. faecium*), having smaller cell size and being easier to produce, are much more resistant than lactobacilli to pelletizing. This may be one reason why most commercial probiotics to date are enterococci.[15]

Pollmann and Bandyk[142] determined the stability of three commercial *Lactobacillus* products in nonmedicated and medicated (lincomycin) piglet starter feed stored for 3 months in different environments. The samples stored in a refrigerator maintained their stability relatively well during the trial. However, the activity of the samples stored in a pig nursery dropped substantially within the first week, and at the end of the trial there was no viability left in some samples. It was also noted that activity loss was slightly greater in the medicated feed.

Alaeddinoglu et al.[143] studied the activity-loss kinetics of freeze-dried *Lactobacillus* cultures and pointed out the importance of optimizing the type and concentration of cryoprotectants during drying. Kearney et al.[144] improved viability of

L. plantarum inoculum after lyophilization and rehydration by immobilizing the cells in calcium-alginate beads containing cryoprotectants. Many other attempts (e.g., microencapsulation) have been made to improve survival of probiotic preparations, and some have been successful.[145]

F. Mixed Probiotics

Although the probiotic concept is theoretically a sound method for supporting animal performance without antibiotics, it is not simple to introduce the right bacteria strains at the right time to the right animal in a biologically and economically efficient way. Another way of thinking is to find substrate(s) that could create a selective pressure on the normal gut microbiota, especially in the large intestine (prebiotics). This means that the animals select their beneficial microbes in situ.

Lactulose and lactitol are synthetic disaccharides made industrially from lactose by several isomerization and hydrogenation steps, respectively. The molecules of lactulose and lactitol are composed of galactose with fructose and sorbitol, respectively, connected with a specific β-galactosidic linkage. Many studies suggest (reviewed by Harju[146]) that small intestinal β-galactosidases of mammalian origin split the linkage inside the lactulose or lactitol molecules very little. As a consequence, lactulose and lactitol escape small intestinal digestion and absorption and are fermented by colonic bacteria into organic acids and gases, which lowers the colonic pH and ammonia content. This fermentation may also cause a positive shift in the colonic microbiota, because enzymes of certain strains of lactic acid and bifidobacteria have been shown to degrade lactulose and lactitol better than those of coliforms and clostridia.[147] We have extensively studied the possibility of using these sugars as probiotics in piglets and calves as such, as a mixture, or in combination with selected strains of lactobacilli and enterococci.[148,149] Such mixed pre- and probiotics (synbiotics) may provide several advantages compared to simple usage of LAB strains: (a) the effect of the treatment extends to the whole tract; (b) the preparations are not too sensitive to the feed manufacturing processes; and (c) the competition force of the probiotic strains can be improved by screening for their ability to use nonabsorbable sugars as carbon sources. According to the data available, these sugar-LAB combinations appear to have an advantage compared to simple LAB probiotics in the treatment of young farm animals.

V. THE REGULATORY STATUS OF ANIMAL PROBIOTICS

While the use of conventional, genetically nonmodified LAB in various human applications has not been particularly regulated, the situation is different with microorganisms intended as animal feed additives. The regulatory situation has been reviewed by Feord.[150] In the United States the situation appears to be relatively simple. The so-called directly fed microbials permitted for use are listed by the Association of American Feed Control Officials (AAFCO) and reviewed by the FDA Center for Veterinary Medicines. In the European Union (EU) there exists a thorough regulatory framework to ensure both the efficacy and safety of microorganisms intended as feed additives. The relevant directives are Council Directive 70/524/EEC and 87/53/EEC, the latter defining the guidelines for the assessment procedures. Further specifications for the authorization process are defined in additional directives 93/113/EEC and 94/40/EEC. The assessment guidelines are regularly reviewed and updated by the Scientific Committee on Animal Nutrition (SCAN).

Since 2003 SCAN has been replaced by the Panel on Additives and Products or Substances in Animal Feed in connection with the European Food Safety Authority (EFSA). For permanent authorization both efficacy and safety of the additive must be established, while only demonstration of safety is necessary for a temporary authorization for products already on the market.

Safety assessment includes safety for the target species, the user, and the consumer. For the target species safety is demonstrated by a tolerance test using a 10-fold overdose for a minimum of one month (depending on species and animal category). Irritancy and sensitization tests must be performed to ensure the occupational safety aspects, while genotoxicity tests and 90-day toxicity studies are required to prevent undefined potentially harmful microbial products from entering into the food chain. These latter requirements are relatively new (established in 2001), and it remains to be seen how they can be implemented in practice. An aspect that has received much attention and detailed guidelines from SCAN is the exclusion of transmissible antibiotic resistance markers from microorganisms intended as animal feed additives.

The microbial preparations so far temporarily approved in the EU are listed in Table 7.

VI. ZOOTECHNICAL TRIALS WITH LAB AS PROBIOTICS

A. Pigs

The three main probiotic types tested in pigs are nonviable and viable *Lactobacillus* (mainly *L. acidophilus*) or viable *Enterococcus faecium.* The probiotics were mixed in the diet (starter piglets, growing-finishing pigs) at the rate of 10^4-10^7 CFU/g, or the liquid diet (milk) was fermented with the probiotic strain. In very few cases more than one strain was used and most of the strains were lacking host specifity. Of the 39 piglet trials summarized in Table 8, 25 positive responses over the control were obtained with probiotics, but a significant response were recorded only in 9 trials ($p < 0.05$). In contrast, negative results were monitored in 11 trials, 4 being statistically significant at the 5% level. Feed conversion was improved in 12 trials, and in 10 trials probiotic-treated piglets showed worsened feed efficiency. Decrease in feed efficiency has been often associated with fermentation of milk.

Far fewer zootechnical trials with LAB as probiotics have been made with growing-finishing pigs, which is not surprising because adult pigs digest their feed better, have improved immunity, and are more resistant to intestinal disorders than young piglets. It appears, however, that slight improvements in performance can be obtained also in adult pigs, but the responses (negative or positive) are of lower magnitude than in piglet trials (Table 9). From Tables 8 and 9 it is not possible to conclude whether *Lactobacillus* products are better than *Enterococcus*, or vice versa.

In the trials conducted in our laboratory, the use of host-specific strains of *L. fermentum* and *E. faecium* alone or in combination with lactulose and lactitol for piglets has been tested on several commercial farms. In five of the seven trials summarized in Table 10, the probiotic treatments gave better responses than controls in terms of improved daily gain, although in trials 3–7 a chemical growth promoter was included in the diet. Overall improvement was about 5.5% if all trials are evaluated together. Consequently, a slight decrease in mortality was recorded, the mean values being 7.7 and 9.9% for the probiotic and control groups, respectively. According to these data, it can be concluded

Table 7 Probiotic Preparations with Temporary Authorization as Feed Additives

Microorganism	Registration number	Species or category of animals
Bacillus cereus var *toyoi*	NCIMB 40112	Chickens for fattening, laying hens
	CNCM I-1012	Calves, cattle for fattening
		Breeding does
		Rabbits for fattening
		Piglets, sows[a]
Saccharomyces cerevisiae	NCYC Sc 47	Rabbits for fattening
		Sows, piglets
		Dairy cows
Saccharomyces cerevisiae	CBS 493.94	Calves, cattle for fattening, dairy cows
Saccharomyces cerevisiae	CNCM I-1079	Sows, piglets
Saccharomyces cerevisiae	CNCM I-1077	Dairy cows, cattle for fattening
Enterococcus faecium	ATCC 53519	Chickens for fattening
	ATCC 55593	
Pediococcus acidilactici	CNCM MA 18/5M	Chickens for fattening
		Piglets, piglets for fattening
Enterococcus faecium	NCIMB 10415	Chickens for fattening
		Pigs for fattening, sows, piglets
		Cattle for fattening, calves
Enterococcus faecium	DSM 5464	Piglets
		Chickens for fattening
		Calves
Lactobacillus farciminis	CNCM MA 67/4R	Piglets
Enterococcus faecium	DSM 10663/NCIMB	Piglets
	10415	Calves
		Chickens for fattening
Saccharomyces cerevisiae	MUCL 39885	Piglets
		Cattle for fattening
Enterococcus faecium	NCIMB 11181	Calves
		Piglets
Enterococcus faecium	DSM 7134	Calves
Lactobacillus rhamnosus	DSM 7133	Piglets
Lactobacillus casei	NCIMB 30096	Calves
Enterococcus faecium	NCIMB 30098	
Enterococcus faecium	CECT 4515	Piglets
		Calves
Streptococcus infantarius	CNCM I-841	Calves
Lactobacillus plantarum	CNCM I-840	
Bacillus licheniformis	DSM 5749	Piglets[b]
Bacillus subtilis	DSM 5750	Sows, pigs for fattening
		Chickens for fattening
		Turkeys for fattening
		Calves
Enterococcus faecium	DSM 3530	Calves

[a]Authorization without a time limit (12.2.2002).
[b]Authorization without a time limit (4.11.2000).
Source: European Commission on Health and Consumer Protection, November 2002.

Table 8 Effect of *Lactobacillus* and *Enterococcus* Probiotics on Performance of Unweaned Sucking and Weaned Starter Piglets

		Performance (% of control)		
Type and dosage of probiotic	Animals	Gain	Feed/gain	Refs.
L. acidophilus in feed	Starter fed	+10.8	−7.2	[159]
L. acidophilus in feed	Starter fed	No resp.	No resp.	[160]
L. acidophilus, nonviable	Starter fed	+4.7	−6.4[a]	[161]
L. acidophilus, 4×10^6 CFU/g feed	Starter fed	+7.2	±0.0	[99]
L. acidophilus, 750 mg/kg (trial 1)	Starter fed	+4.5	−6.7	[162]
L. acidophilus, 750 mg/kg (trial 2)	Starter fed	+9.7	−21.4[a]	
S. faecium[c] 1250 mg/kg (trial 2)	Starter fed	−7.6	−8.7[a]	
L. acidophilus, 4×10^6/kg	Starter fed	−1.6	±0.0	[140]
L. acidophilus, nonviable				
Trial 1	Milk replacer fed	−8.2	+4.0	[163]
Trial 2		−6.8[a]	+9.9	
S. faecium, 10^6 (per os)				
Single	0–3 weeks	+0.8	+39.8[a]	[127]
3 Days continuous	Milk replacer fed	−8.3 to +1.3	−9.5 to +11.2[a]	
		−5.7 to −1.6	+0.0 to +3.0	
S. faecium, 10^6/g feed	Sucking	+9.7	No data	[164]
L. fermentum, 10^9/d (host-specific)	0–9 weeks	−7.1	No data	[139]
S. faecium, 2×10^8 CFU/d	Starter fed	11.1[a]	No data	[165]
L. bulgaricus + *S. thermophilus*	Fermented milk	−8.8	+18.6[a]	[93]
L. bulgaricus + *S. thermophilus*	Fermented milk	−21.0[a]	+19.5	
L. reuteri, host-specific	Fermented milk	−22.5[b]	+48.3	
S. faecium, 10^6 CFU/g	Starter fed	+2.3	−1.8	[166]
Antibiotic		+6.3	±0.0	
10^6 CFU/g + antibiotic		+4.0	±0.0	
S. faecium, 10^6 CFU/g				
Trial 1	Starter fed	−15.4	No data	[167]
Trial 2	Starter fed	+23.3	−10.4	
L. acidophilus, nonviable (0.1%)	Starter fed	+10.4[a]	±0.0	[131]
S. faecium, 10^6/g feed	Starter fed	Improved	Improved	[168]
S. faecium, 10^6/g feed		+13	+2	
Antibiotic		+24	+11	
Antibiotic + *S. faecium*		+33	+11	
L. acidophilus, 10^9 CFU + *S. faecium*, 10^9 CFU, 3 days orally postpartum	Sucking	+14.3[a]	No data	[17]
L. acidophilus, 3×10^9 CFU/d	Sucking	+20[a]	No resp.	[169]
	Weaning	+8[a]		
B. pseudolongum, 3×10^8 CFU/d	Sucking	+18[a]	−8[a]	
	Weaning	+9[a]		
E. faecium, 1.1×10^7 CFU/g feed	Sucking	+3.6	No data	[170]
E. faecium, 7.3×10^6 CFU/g feed	Rearing	+5.6	+2.4	
E. faecium, 1.3×10^{10} CFU/d	Sucking	No resp.	No resp.	[171]

(*continued*)

Table 8 Continued

Type and dosage of probiotic	Performance (% of control)			Refs.
	Animals	Gain	Feed/gain	
E. faecium, 3.7×10^9 CFU/d	Sucking	No resp.	No resp.	
E. faecium + *L. acidophilus*, 5×10^7 CFU + *S. cerevisiae* 2×10^7 CFU	Weaning	+8	No data	[172]
L. reuteri, 2×10^6 CFU/d	Sucking	+45[b]	−34[b]	[173]
L. reuteri, 2×10^8 CFU/d	Sucking	+31[b]	−34[b]	

[a]Statistically different compared to control ($p < 0.05$).
[b]Statistically different compared to control ($p < 0.01$).
[c]*Streptococcus faecium* is currently named *Enterococcus faecium*. The older designation is still used in the reference.

that mixed probiotics are superior to simple ones. In trial 3/1991, all the piglets were treated with sulfa just after weaning, which evidently weakened the barrier effect of indigenous microbiota and greatly improved the competitiveness of probiotic bacteria. Markedly better weight gain and lower mortality in the probiotic group could in this particular trial be explained by the competitive exclusion concept.

B. Calves

Table 11 summarizes the results of 17 calf trials conducted between 1978 and 2001 testing the effectiveness of LAB probiotics. Twenty-five experiments showed positive results if judged by the daily gain figures, and in 6 cases the improvement in gain was significant.

Table 9 Effect of *Lactobacillus* and *Enterococcus* Probiotics on the Performance of Growing Finishing Pigs

Type and dosage of probiotic	Performance (% of control)			Refs.
	Animals	Gain	Feed/gain	
L. acidophilus in feed	Growing-finishing	+8.4	−5.8	[159]
L. acidophilus, nonviable	Growing-finishing	+4.3	−0.7	[161]
L. acidophilus, 750 mg/kg	Growing-finishing (35–95 kg)	−1.2	−0.9	[162]
S. faecium, 1250 mg/kg		−1.2	−0.9	
L. acidophilus, 4×10^6/kg (trial 1)	Growing-finishing	−5.8[a]	+3.1	[140]
L. acidophilus, 4×10^6/kg (trial 2)	(17–100 kg)	+1.3	±0.0	
L. acidophilus	Growing-finishing	Improved[a]	No response	[174]
S. faecium		Improved[a]	No response	
S. faecium, 10^6/g feed	Growing-finishing	Improved	Improved	[175]

[a]Statistically different compared to control ($p < 0.05$).

Table 10 Effect of Host-Specific *Lactobacillus* and *Enterococcus* Probiotics on the Performance of Small Piglets

Trial/year	No. of animals, age (weeks)	Probiotic type and dosage	Growth response (% of control)	Mortality (treated vs. control)
1/1987	108, 0–6	*E. faecium* + *L. fermentum* (10^9 CFU/d) Lactulose + lactitol (top-dressed)	+14.2[b]	0.0 vs. 1.1
2/1987	102, 0–5	*Lactobacillus sp.* (10^9 CFU/d) Top-dressing to starter	+1.8	3.9 vs. 6.8
3/1991	98, 0–11	*E. faecium* + *L. fermentum*, 10^6–10^7 CFU/g starter	+15.0[b]	6.8 vs. 15.9
4/1991	102, 0–11	*E. faecium* + *L. fermentum*, 10^6–10^7 CFU/g starter	+ 5.5	6.6 vs. 10.7
5/1991	189, 0–10	*E. faecium* + *L. fermentum*, 10^6–10^7 CFU/g starter	−5.0	11.6 vs. 12.6
6/1991	106, 0–11	*E. faecium* + *L. fermentum* (10^9 CFU/d) Lactulose + lactitol (top-dressed)	−3.5	15.6 vs. 19.5
7/1991	130, 0–10	*E. faecium* + *L. fermentum*, 10^6–10^7 CFU/g starter	+4.7	9.7 vs. 2.7
8/1991	50,[c] 0–7	*E. faecium* + *L. fermentum* (10^9 CFU/d) Lactulose + lactitol	+11.2[b]	
		L. fermentum (10^9 CFU/d) + *L. acidophilus* (10^9 CFU/d)	+6.0	

[a]Trials conducted on commercial farms between 1987 and 1991 in the authors' laboratory.
[b]Statistically different compared to control ($p < 0.05$).
[c]Agricultural Research Centre, Swine Research Station.[176]
Note: In trials 3–7 both the control and probiotic diets were pelletized and medicated (50 ppm carbadox) commercial creep starters.

Interestingly, the overall 11.4% improvement is of the same magnitude as in the reviewed piglet trials (see Table 8). It is also impossible to compare the efficiencies of *Lactobacillus* and *Enterococcus* products, because in many cases the exact nature of the treatment was not reported.

C. Ruminants

The addition of LAB to the diet of adult ruminants has been investigated in only a few trials (Table 12). The complex ruminal microbiota forms a barrier that can overcome the probiotic strain, and therefore the effects in rumen fermentation are variable and difficult to interpret.

McCormick[151] observed changes in rumen fermentation in one trial, but not in another, when steers were treated with *L. acidophilus.* Variable data were also presented by Hoyos et al.[152] and Rust et al.[153] about the performance of dairy and beef cattle, respectively.

In sheep the use of probiotic yeasts has been shown to improve fermentation efficiency[154] and the production of volatile fatty acids.[155] These beneficial effects are attributed to the oxygen-scavenging ability of yeasts.

Table 11 Effect of *Lactobacillus* and *Enterococcus* Probiotics on the Performance of Calves

Type and dosage of probiotic	Performance (% of control)			
	Animals	Gain	Feed/gain	Refs.
L. acidophilus + *L. lactis* (ferm. milk)	Small calves (1–5 wk)	−11.6	No response	[177]
L. acidophilus, 10^6 CFU/L milk	Small calves (0–3 wk)	No response	No response	[178]
L. acidophilus	Young bulls	No response	No response	[179]
Lactobacillus fermentation product	Small calves (0–8 wk)	+5.3	No response	[97]
	Small calves (0–10 wk)	+28.3[a]	−1.5	
Lactobacillus fermentation product	Weaned calves (28 days)	−8.5	No response	[180]
Killed *Lactobacillus*	Weaned calves (35 days)	No response	Impaired	[181]
	Transported calves (28 days)	Improved	Improved	
Viable *Lactobacillus*	Transported calves (28 days)	No response	No response	[182]
E. faecium, 0.5×10^7/g diet	Small calves	Improved[a]	Improved[a]	[183]
Lactobacillus sp.	Small calves (0–21 days)	−0.3	No data	[184]
E. faecium, 1.0×10^7/g diet	Small calves	No response	No response	[185]
L. acidophilus, 10^9–10^{10} CFU/d/calf	Small calves (0–7 wk)	−7.6	+1.8	[137]
E. faecium, 10^6 CFU/g in MR	Small calves (0–8 wk)	+4.1	−7.6	[186]
L. acidophilus, 10^6 CFU/g in MR	Small calves (0–10 wk)	+0.5	−4.0	
E. faecium, 10^{10} CFU/g in MR (0–5 days)	Small calves (30 days)	+20[a]	No data	[17]
Lactobacillus sp, $0.8–8.0 \times 10^6$ CFU/g in MR	Small calves	+6–7	−4–5	[19]
Enterococcus sp, $0.8–8.0 \times 10^6$ CFU/g in MR	Small calves	+3–4	−23	
L. fermentum[b] + *L. delbrueckii*[b] + lactitol	Transported calves (4–10 wk)	+15	No data	[176]
L. acidophilus + *S. faecium* (viable), 10^9 CFU	Young dairy calves (29–44 days)	+15	+2	[187]
L. acidophilus (nonviable), 10^8 CFU/day	Young dairy calves (2–64 days)	+15	+12.5	

(*continued*)

Table 11 *Continued*

Type and dosage of probiotic	Performance (% of control)			Refs.
	Animals	Gain	Feed/gain	
L. plantarum + *P. cerevisiae*, 5×10^{10} CFU/g, 10^5 CFU/g	Bull calves (1–6 wk)	−6.5	+1.6	[188]
L. acidophilus, 3×10^9 CFU/d	Calves (1–8 wk)	+21[a]	−12.7[a]	[169]
B. thermophilum + *E. faecium* + *L. acidophilus*	Calves (1–8 wk)	+11.9	−3	
B. pseudolongum	Calves (1–8 wk)	+25[a]	−11.4[a]	
L. acidophilus + *L. plantarum*	Small calves (0–12 wk)	+5	−4	[189]
L. acidophilus 27SC, 1.85×10^7 CFU/L	Small calves (0–12 wk)	+18	−14.6	
L. acidophilus, 10^8 CFU/day in MR	Bull calves (0–6 wk)	+7	No response	[190]
L. acidophilus, 4×10^6 CFU/g	Bull calves (1–11 wk)	+3	−1	[191]
L. acidophilus + *L. casei* + *L. plantarum* + *Str. faecium* 4×10^7 CFU/g		10	−4	
Str. faecium, 4×10^{10} CFU/g		6	−4	
B. bifidum, 4×10^{10} CFU/g		9	±0	
E. faecium + *L. acidophilus* (8.8×10^8 CFU) + *S. cerevisiae* (8.8×10^8 CFU)	Calves (3 d–6 wk)	+30[c]	+7.8	[172]
E. faecium + *L. acidophilus* (8.8×10^8 CFU) + *S. cerevisiae* (8.8×10^8 CFU) + FOS	Veal calves (1–6 wk)	+1.6	−2.3	
L. acidophilus + *L. jugarti* + *L. casei*, 10^9–10^{10} CFU/d	Calves (1–8 wk)			[192]
	Grain diet	+2.3	−18	
	Grainless diet	±0	−10.7	

[a]Statistically different compared to control ($p < 0.05$).
[b]10^9 CFU/d.
[c]Statistically significantly different compared to control ($p < 0.01$).
MR = milk replacer.

Thus, the most promising area of the digestive tract of ruminants for the function of probiotics appears to be the reticulorumen. As indicated by McCormick,[151] changes in rumen fermentation pattern and digestion can be obtained by feeding of LAB. Moreover, the massive microbial population in the rumen greatly affects the energy and protein utilization and hence the performance of the ruminant.

The role of LAB in the lower digestive tract of ruminants is difficult to estimate because very few data are available. It is possible that a ruminant reacts to fermentation in the intestine in a different way than do nonruminant animals. For example, preliminary data in our laboratory indicate that fermentation products (lactic acid, VFA) in the hindgut could decrease feed intake by the ruminant.

Treatment	Advantage	Ref.
L. acidophilus [a]	Variable changes in acetic/propionic-ratio in rumen VFA	[151]
L. acidophilus + [b]	Lowered acetic/propionic ratio, higher VFA and fiber digestion	[151]
L. acidophilus + *E. faecium* + *S. cerevisiae* [c]	Increased milk yield and milkfat content	[152]
L. acidophilus *Bifidobacterium* sp. *L. faecalis* [d]	No advantages in feed intake, daily gain, or health (beef cattle)	[153]
L. lactis L. acidophilus *B. subtilis* [e]	No advantages in feed intake, daily gain, or health (beef cattle)	[153]
L. acidophilus [f]	Statistically significantly ($p > 0.01$) higher daily gain and better feed intake, no beneficial effects under stress	[193]

[a] 2×10^7 CFU/animal/day.
[b] 2×10^7 CFU/animal/day.
[c] Dosage not reported.
[d] 6×10^9 CFU/animal/day.
[e] 3.3×10^9 CFU/animal/day.

VII. CONCLUDING REMARKS

The probiotic concept and the components of competitive exclusion have been demonstrated in many studies using, e.g., gnotobiotic models or specific in vitro techniques, in a scientifically sound way. However, the results obtained from animal trials testing the effect of LAB probiotics on the growth and health of farm animals have been quite variable. The overall efficiency of probiotics to date does not reach the level that can be obtained with feed antibiotics, although in some trials positive effects can be noted when LAB are added to medicated feed.

One reason for the variable results with probiotics in field trials is certainly the complexity of the phenomenon itself. No improvements in performance can be expected when animals are equipped with a well-functioning gut microbiota adapted to grow in beneficial symbiosis with the host. In contrast, in the presence of any kind of environmental stress (management methods, diet) causing imbalance in the intestinal ecosystem, high-quality probiotics certainly have a potential to boost animal performance. On the other hand, the properties and dosing methods of the preparations used in trials may not have been in line with basic probiotic concepts (e.g., probiotic dosages too low to overcome the barrier effect of the indigenous microbiota or using the wrong types of bacteria). Additionally, the sensitivity of the isolated gut bacteria to the industrial cultivation and the processes of compound feed technology can be a real problem. It has been suggested that LAB may lose their adhesion ability due to disappearance of plasmids during long-term technological usage. Therefore, much attention has to be paid to improving the tolerance and viability of the probiotic bacteria, especially the most promising *Lactobacillus* strains.

The use of synbiotics—a combination of probiotic strains and nonabsorbable sugars—could improve the efficacy of probiotic therapy.[156] Future experience will test

the validity of this method as a tool for solving the problems stated above and for obtaining more consistent responses with probiotics in commercial circumstances.

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21

Lactic Acid Bacteria in Fish and Fish Farming

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I. LACTIC ACID BACTERIA IN FISH DIGESTIVE TRACT

It has been well documented in several investigations that lactic acid bacteria (LAB) are a part of the native microbiota of aquatic animals from hatching onwards (Table 1). The great variation in the frequency of LAB reported in the different studies quoted in Table 1 may reflect that real variation exists between fish species, but these results may also reflect the shortcomings of the methods used to select for and isolate these bacteria. Furthermore, geographical location must also be taken into consideration.

In their recent review on probiotic bacteria as biological control agents in aquaculture, Verschuere and coworkers[1] discussed that because LAB normally account for only a marginal part of the intestinal microbiota of fish, it can be questioned if bacteriocins produced by LAB can effectively contribute to the health status of aquatic animals. The main reason for not isolating LAB from aquatic animals might be that these LAB from fish are generally slow-growing microorganisms; Ringø and Gatesoupe[2] recommended an incubation time of up to 4 weeks at low temperature (4 and 12°C). In addition, as for the human gastrointestinal tract, it can also be expected that a number of LAB representatives are not culturable by existing methods.

Before discussing which taxa may or may not be associated with the digestive tract of fish, it is essential to present some information about how LAB isolated from fish have been identified. In most of the investigations cited in Table 1, the classic approach based on morphological and physiological features were used. However, recently several molecular methods, including 16S rDNA,[3–9] ribosomal RNA (rRNA) (Ringø and Sperstad, unpublished results), amplified fragment length polymorphism (AFLP),[6,8,9]

Table 1 Lactic Acid Bacteria Isolated from Aquatic Animals

Source		Lactic acid bacteria isolated from: Whole intestinal tract	Stomach	Small intestine	Large intestine	Feces	Gills	Refs.
Lactobacillus spp.								
Arctic charr	A		+			+		[33]
	A			+	+			[34]
	A					+		[36]
	A					+		[35]
	A		+	+	+	+		[4]
	A			+	+	+		[28]
Atlantic cod		+						[114]
Atlantic salmon		+						[25]
	A			+	+			[7]
	A	+	+	+				Ringø and Bendiksen, unpublished
Brown trout[a]								[14]
Herring		+						[12]
		+						[115]
Various fish		+						[13]
Lb. plantarum–like								
Arctic charr	A		+	+	+	+		[4]
Brown trout[a]								[14]
Saithe	A	+						[15]
Carnobacterium spp.								
Arctic charr	A		+	+	+	+		[4]
	A				+			[16]
	A				+			[9]
Brown trout[a]								[14]
Rainbow trout		+						[18]

		+				[19]
	J	Intestinal contents				[17]
	A			+	+	Ringø unpublished
Various fish		Intestinal contents				[20]
C. divergens–like						
Arctic charr	A				+	[22]
	A			+	+	[16]
	A			+		[17]
Atlantic cod	J/A			+	+	[21]
	L	+[b]				[23]
	A				+[c]	[10]
Atlantic salmon	A			+	+	[21]
Brown trout[a]						[14]
Saithe	A		+	+		[21]
Wolffish	F	+				[8]
C. funditum–like						
Arctic charr	A				+	[9]
C. gallinarum–like						
Arctic cod	A				+[c]	[10]
C. inhibens						
Atlantic salmon		+				[24]
Atlantic cod	A				+[c]	[10]
C. mobile–like						
Arctic charr	A				+	[16]
Atlantic cod	A				+[c]	[10]
Atlantic salmon	A				+	Ringø and Sperstad, unpublished
Rainbow trout	A				+	Ringø and Sperstad, unpublished

(*continued*)

Table 1 Continued

Source		Lactic acid bacteria isolated from: Whole intestinal tract	Stomach	Small intestine	Large intestine	Feces	Gills	Refs.
C. piscicola–like								
Arctic charr	A		+	+	+	+		[4]
	A			+				[16]
Atlantic cod	A			+	+			Strøm and Ringø, unpublished
	A					+		Ringø, unpublished
	A				+[c]			[10]
Atlantic salmon	A			+	+			[7]
	A						+	[6]
	J				+			Ringø, unpublished
	J			+	+	+		Strøm and Ringø unpublished
Brown trout[a]								[14]
Fish[a]		+						[117]
Rainbow trout		+						[118]
Saithe	A			+	+			Strøm and Ringø, unpublished
Turbot	J		+					Ringø, unpublished
Aerococcus spp.								
Atlantic salmon		+						[25]
Enterococcus spp.								
Brown trout[a]								[14]
Common carp		+						[26]
E. durans–like								
Brown trout[a]								[14]

E. faecium–like							
Common carp/prawn		+					[26]
E. gallinarum–like							
Atlantic salmon	A				+		Ringø and Sperstad, unpublished
E. pseudoavium–like							
Atlantic salmon	A				+		Ringø and Sperstad, unpublished
E. faecium–like							
Common carp		+					[26]
E. gallinarum–like							
Atlantic salmon	A				+		Ringø and Sperstad, unpublished
E. pseudoavium–like							
Atlantic salmon	A				+		Ringø and Sperstad, unpublished
Lactococcus spp.							
Brown trout[a]							[14]
Common carp		+					[26]
Lac.garvieae–like							
Common carp		+					[26]
Lac. lactis–like							
Rotifer[d]							[27]
Leuconostoc spp.							
Arctic charr	A					+	[28]
Leu. mesenteroides–like							
Arctic charr	A		+	+	+		[4]
Pediococcus acidilactici–like							
Common carp		+					[26]
Fish[a]		+					[29]

(*continued*)

Table 1 Continued

Source		Lactic acid bacteria isolated from: Whole intestinal tract	Stomach	Small intestine	Large intestine	Feces	Gills	Refs.
Streptococcus spp.								
Arctic charr	A		+			+		[33]
	A			+	+			[34]
	A					+		[35]
	A				+	+		[28]
	A		+	+	+			[4]
	A			+	+			[16]
Atlantic salmon	A			+	+			[7]
Carp		+						[119]
Eel, European		+						[120]
Eel, Japanese		intestinal content						[121]
Goldfish		+						[122]
Rainbow trout		Intestinal content						[121]
Various salmonids		+		+	+			[31]
Turbot	J		+	+	+			Ringø, unpublished
Yellowtail		+						[123,124]
Giant freshwater prawn	L	+						[125]
Vagococcus spp.								
Brown trout[a]								[14]
Weissella hellencia								
Flounder		+						[84]

[a]No further information given.
[b]Reclassified from *Lb. plantarum* to *C. divergens*.[8]
[c]Isolated from the hindgut chamber.
[d]Isolated from whole animal.
A, adult; J, juvenile; F, fry; L, larvae.

DNA-DNA similarity,[9] and random amplification of polymorphic DNA (RAPD),[10] have become important tools in the identification of LAB from fish.

A. Lactobacilli

Some of the gut *Lactobacillus* spp. cited in the literature and therefore here probably belong to the genus *Carnobacterium.* Differentiating lactobacilli from carnobacteria can be done according to the following key characteristics: growth on acetate agar (pH 5.4), growth at pH 4.5, growth on CTAS (cresol thallium acetate sucrose) agar (pH 9.1), presence of *meso*-diaminopimelic acid in the cell wall, lactic acid isomers produced, major $C^{18:1}$ in the cell wall, GC content, and PCR.

Knowledge about lactobacilli on the skin and gills and in the gut of fish was first reported by Dyer in 1947[11] in a study of Atlantic cod (*Gadus morhua* L.). Fourteen years later, Kraus[12] isolated lactobacilli from herring (*Clupea harengus* L.). Kvasnikov and coworkers[13] described lactobacilli as a normal part of the microbiota of Cyprinidae, Escocidae, and Percidae at the larval, fry, and fingerling stages. Furthermore, the authors demonstrated seasonal variations of lactobacilli in fingerlings of pond fish such as *Cyprinus carpio*, *Aristichthys nobilis*, and *Hypophthalmichthus molitrix*, with the highest population levels in midsummer. Today, it is well documented that lactobacilli are part of the native intestinal microbiota of Arctic charr (*Salvelinus alpinus* L.), Atlantic cod, Atlantic salmon (*Salmo salar* L.), and brown trout (*Salmo trutta*) (Table 1). However, lactobacilli do not seems to belong to the dominant microbiota in fish. Gonzalez et al.[14] reported that typical lactobacilli accounted for only 0.44% of the LAB isolated from the intestines of wild brown trout. Three of the investigations cited in Table 1 demonstrated the presence of *Lactobacillus plantarum*–like strains isolated from the alimentary tracts of Arctic charr,[4] saithe (*Gadus virens* L.),[15] and brown trout.[14]

B. Carnobacteria

The presence of carnobacteria in the digestive tract of fish is well documented (Table 1). In some of these studies, *Carnobacterium* spp. were reported in hatchery-reared Arctic charr,[4,16,17] rainbow trout (*Oncorhynchus mykiss* Walbaum),[5,18,19] brown trout,[14] and various fish.[20] Some of these strains probably belong to already known strains. However, as no information beyond biochemical characterization has been done, molecular methods are needed for correct identification.

The first study demonstrating the presence of *Carnobacterium divergens*–like strains in fish was reported by Strøm.[21] She found that the bacterial species was dominant in the gastrointestinal tract (small and large intestine) of juvenile Atlantic salmon, juvenile/adult Atlantic cod, and saithe. Later investigations isolated *C. divergens*–like strains in the digestive tract of adult Arctic charr,[9,16,22] larvae of Atlantic cod,[23] large intestine and hindgut chamber of adult Atlantic cod,[10] as well as in brown trout[14] and common wolffish (*Anarhichas lupus* L.).[8]

Isolates identified as *Carnobacterium funditum*–like have only been obtained from the large intestine of Arctic charr fed linseed oil prior to challenge with the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida*.[17] One recent study demonstrated *Carnobacterium gallinarum*–like strains from large intestine and hindgut chamber of Atlantic cod.[10] The presence of *Carnobacterium inhibens* has been reported in the alimentary tracts of Atlantic salmon[24] and Atlantic cod.[10] Only one *Carnobacterium*

species is motile—*Carnobacterium mobile*. Two recent studies reported that *C. mobile*–like strains colonize the digestive tract of Arctic charr[16] and Atlantic cod.[10] *Carnobacterium piscicola* has been isolated from the gastrointestinal tract, feces, and gills of several fish species (Arctic charr, Atlantic cod, Atlantic salmon, brown trout, rainbow trout, saithe, and turbot) (Table 1).

C. *Aerococcus*

Aerococcus–like strains are rarely isolated from aquatic animals, and only one study has reported their presence in fish gut.[25] Five strains were isolated from Atlantic salmon, but the authors did not present information about the population level, as this study focused on the antagonistic activity of gut bacteria.

D. *Enterococcus*

Bacteria of the genus *Enterococcus* have been isolated from the intestine of common carp (*Cyprinus carpio*) and freshwater prawns (*Macrobrachium rosenbergii*),[26] brown trout,[14] and Atlantic salmon (Ringø and Sperstad, unpublished data). In common carp, *Enterococcus faecium* were isolated but in small numbers.[26] These authors suggested that these strains might have a probiotic potential in fish, as the strains could grow at pH 9.6, in 6.5% NaCl, 40% bile, and at high cultivation temperature (50°C). However, this controversial hypothesis must be tested in future studies.

In their study on freshwater fish, mainly wild brown trout, Gonzalez and coworkers[14] isolated 249 strains of LAB, of which eight strains belong to the genus *Enterococcus* (six *E. durans*–like strains and two *Enterococcus* spp.).

E. *Lactococcus*

Lactococcus strains have been isolated from some aquatic animals.[14,26,27]

F. *Leuconostoc*

Bacteria of the genus *Leuconostoc* have been isolated in two investigations.[4,28] In their study with Arctic charr, Ringø and Strøm[28] showed that approximately 4.5% of the viable counts in feces of fish fed capelin roe diet belong to *Leuconostoc.* In a later study with Arctic charr fed different polyunsaturated fatty acids, Ringø and coworkers[4] isolated several adherent *Leuconostoc mesenteroides*–like strains from stomach, small, and large intestine. However, in this study *Leu. mesenteroides* was not isolated from feces.

G. *Pediococcus*

In fish, *Pediococcus acidilactici*–like strains have been isolated in only two studies.[26,30] This species may present some probiotic features, such as bacteriocin production,[30] and recently it was used in combination with *Saccharomyces cerevisiae* in the enrichment process of *Artemia nauplii*.[30]

H. *Streptococcus*

Bacteria of the genus *Streptococcus* was initially described as a part of the gut microbiota in fish by Trust and Sparrow.[31] Since then, the genus has been isolated from stomach, small, and large intestine of several fish species, and prawn (Table 1).

I. *Vagococcus*

In fish, this bacterial genus has only been isolated from fish, brown trout, in one study.[14]

J. Aquatic Habitats of LAB

All reports on the presence of LAB in fish from both freshwater and marine environments suggest that the fish gut may be a natural reservoir for LAB. Other habitats may be other marine animals, sediments, and water. Lactic acid bacteria, like other nonsporogenous bacteria, can survive in the water column. Franzmann and coworkers[32] isolated *C. funditum* and *C. alterfunditum* from the water of Ace Lake, Antarctica, and Jöborn[3] showed that *C. inhibens* originally isolated from the intestine of Atlantic salmon survived at least 26 days in an artificial seawater solution. These interesting results should encourage microbiologists to carry out further investigations on this topic.

In addition to the numerous investigations demonstrating the presence of LAB in the digestive tracts of several different fish species, several studies have reported on the isolation of LAB (carnobacteria, lactobacilli, lactococci, leuconostocs, and pediococci), from cold-smoked and fermented fish (Table 2).

Table 2 Lactic Acid Bacteria Isolated from Cold-Smoked and Fermented Fish

Source	Lactic acid bacteria	Refs.
Carnobacteria		
Vacuum-packed cold-smoked salmon	*C. piscicola*	[125]
Spoiled cold-smoked salmon	*Carnobacterium* spp.	[127]
Lactobacilli		
Chilled, stretch-wrap-packed Channel catfish	*Lb. plantarum*	[128]
Spoiled cold-smoked salmon	*Lb. curvatus*; *Lb. sakei*; *Lb. plantarum*	[127]
Fermented fish	*Lb. pentosus*; *Lb. plantarum*	[129]
Low-salt fermented fish products	*Lb. pentosus*; *Lb. plantarum*	[130]
Spoiled, vacuum-packaged, cold-smoked rainbow trout	*Lb. plantarum*	[131]
Jeot-gal, a Korean fermented fish food	*Lb. brevis*	[132]
Fermented fish	*Lb. acidipiscis* sp. nov	[133]
Lactococci		
Low-salt fermented fish products	*Lac. lactis* ssp. *lactis*	[130]
Jeot-gal, Korean fermented fish food	*Lac. lactis*	[132]
Leuconostoc		
Spoiled cold-smoked salmon	*Leuconostoc* spp.	[127]
Low-salt fermented fish products	*Leu. citreum*	[130]
Spoiled, vacuum-packaged, cold-smoked rainbow trout	*Leu. citreum*	[131]
	Leu. mesenteroides ssp. *mesenteroides*	
Pediococci		
Low-salt fermented fish products	*P. pentosaceus*	[130]

II. FACTORS AFFECTING LACTIC ACID BACTERIA IN THE DIGESTIVE TRACT

It is well known that LAB are not under normal circumstances numerically dominant in the digestive tract of fish.[2] However, some attempts have been made to increase the level of lactobacilli and carnobacteria colonizing the gastrointestinal tract by nutritional factors such as (a) chromic oxide,[33–35] (b) dietary polyunsaturated fatty acids,[4,36] (c) dietary lipids,[16] (d) dietary lipid sources,[17] and (e) dietary inulin (Ringø, Myklebust, Mayhew, and Olsen, unpublished results).

A. Chromic Oxide (Cr_2O_3)

Chromic oxide has been one of the most widely used indicators for determining nutrient digestibility in fish,[37,38] and until recently it has been assumed that the compound was inert. However, microbial investigations have clearly demonstrated that by feeding the salmonid fish Arctic charr (*Salvelinus alpinus* L.) a diet containing chromic oxide, population levels of lactobacilli in gut and fecal samples remained stable while counts of gram-negative bacteria genera declined.[33–35] Similar observations were made when gram-negative gut isolates and LAB were grown on tryptic soy agar plates with or without supplements of chromic oxide. The reason for the decline in gram-negatives in the gastrointestinal tract has not been elucidated, but Ringø[34] put forward two hypotheses: (a) chromic oxide affects the attachment sites in the gut mucosa/epithelium or (b) oxidase-positive bacteria may be more sensitive to chromic oxide. Unpublished data by Gislason and Ringø showed that supplementation of chromic oxide increased the intestinal activity of the enzyme cholytaurin hydrolase, possibly through the selection of lactobacilli and streptococci, as demonstrated by Ringø.[34]

B. Dietary Polyunsaturated Fatty Acids

Although fatty acids are important in fish metabolism, few studies have evaluated the effect of dietary polyunsaturated fatty acids on the gut microbiota.[4,36] In a study on the effect of linoleic acid (18:2 *n*-6) on intestinal microbiota of Arctic charr, Ringø[36] was unable to isolate LAB in the intestinal contents, but large numbers of *Aeromonas* spp., *Pseudomonas* ssp., and Enterobacteriaceae were isolated when 2.5% linoleic acid was added to a commercial feed. In contrast to these findings, *Lactobacillus* spp. accounted for approximately 10% of the microbiota when the fish were fed the unsupplemented diet. In a recent study with Arctic charr fed casein-based diets supplemented with different fatty acids [18:2 *n*-6, α-linolenic acid (18:3 *n*-3) or a HUFA mix (20:5 *n*-3 and 22:6 *n*-3)], Ringø and coworkers[4] showed no suppression of LAB (*Carnobacterium* spp., *Carnobacterium piscicola*, and *Lb. plantarum*) in the stomach or small or large intestine. However, a significant increase in both total viable counts and population levels of LAB was observed in large intestine and feces of fish fed 7% 18:3 (*n*-3) or 4% HUFA mix. This was due to a large extent to increased contents of *Carnobacterium* spp. The reason for the increase in LAB in fish fed 7% linolenic acid and HUFA mix has not been elucidated, but the authors suggest that dietary fatty acids influence intestinal membrane composition, function, and fluidity, which may affect the attachment sites of the gut mucosa. This controversial hypothesis was later confirmed by Kankaanpää and coworkers.[39] They demonstrated that culturing of Caco-2 cells with arachidonic acid (20:4 *n*-6) reduced the Caco-2 cell adhesion of LAB, whereas 18:3 (*n*-3) did not

interfere adhesion of *Lactobacillus* GG or *Lactobacillus bulgaricus* and promoted the adhesion of *Lactobacillus casei* Shirota.

In view of the results observed by Ringø and coworkers,[4] it is interesting to note that the ability of *C. piscicola*–like isolates to inhibit the fish pathogen *Aeromonas salmonicida* ssp. *salmonicida* was highest in strains isolated from fish fed linolenic acid or the HUFA mix (Ringø, unpublished data). Based on these results it is recommended that greater attention should be paid to increasing the level of intestinal carnobacteria with inhibitory effect against fish pathogens by dietary manipulation. The results obtained from fish fed dietary 18:3 (*n*-3) may lead to the conclusion that it is desirable to increase the level of dietary 18:3 (*n*-3) in commercial diets in order to obtain a higher population level of intestinal strains of *C. piscicola* able to inhibit the growth of *A. salmonicida* ssp. *salmonicida.* However, in this respect it is worthwhile to note that feeding the charr high levels (>15%) of dietary 18:3 (*n*-3) increased accumulation of lipid droplets in the enterocytes and cell damage, which may increase the risk of microbial infections.[40,41]

C. Dietary Lipid Levels

Earlier diets for cultured salmonids contained high amounts of carbohydrates (approximately 20% dry weight), but in recent years there has been a tendency towards decreasing dietary carbohydrate content from about 20 to 10%, with a subsequent increase in the level of dietary lipid from <20 to 30%. Based on this tendency, Ringø and Olsen[16] fed Arctic charr diets containing high (27%) and low (13%) levels of dietary lipid. Dietary manipulation influenced the species composition of carnobacteria, as *Carnobacterium* spp. and *C. mobile*–like strains were only isolated from large intestines of fish fed low dietary lipid, while *C. piscicola*–like strains were isolated from small intestines. *C. divergens*–like isolates were found associated with small and large intestines of fish fed high dietary lipid.

D. Different Dietary Lipid Sources

Vegetable oils stand out as the most likely candidates to substitute in part for marine oils in fish feed, as their total global production is approximately 100 times higher than that of fish oils.[42] This is relevant because commercial aquaculture depends on lipids in feed production, but in the future marine lipids may be limited because of increased fish production. Therefore, finding alternatives to dietary marine lipids has become the focus of research aimed at producing stable supplies of commercial diets at reduced prices. As no information was available about how inclusion of plant oils in commercial raw material affects the gut microbiota of fish, Ringø and coworkers[17] investigated the effect of soybean, linseed, and marine oils on the hindgut microbiota of Arctic charr. This study showed clear differences in the hindgut microbiota of fish fed different oils (after and prior to challenge with *A. salmonicida* ssp. *salmonicida*). Carnobacteria were isolated from the hindgut region of fish fed soybean oil and linseed oil only before challenge, while *Carnobacterium* spp. and *C. funditum*–like strains were isolated from fish fed the same oils after challenge. Furthermore, the ability of carnobacteria to inhibit the growth of *A. salmonicida* ssp. *salmonicida* was highest in strains isolated after challenge. Lødemel and coworkers[43,44] clearly demonstrated that survival of Arctic charr after challenge with *A. salmonicida* ssp. *salmonicida* was improved by dietary soybean oil.

E. Dietary Inulin

The modern concept of prebiotics implies the use of selective agents to favor the growth of protective indigenous gut microbiota. Dietary fiber is a prebiotic that belongs to the broad category of carbohydrates. Burkitt and coworkers[45] defined dietary fiber as "the sum of polysaccharides and lignin which are not digested by the endogenous secretions of the human gastrointestinal tract." They can be classified as soluble (e.g., inulin and oligofructose), insoluble (e.g., cellulose), or mixed (e.g., bran). It is well known from endothermic investigations that dietary fibers are fermented by the anaerobic intestinal microbiota, primarily those colonizing the large intestine.[46–50] This leads to the production of lactic acid and short-chain fatty acids (acetate, propionate, and butyrate) that are utilized by the host[51] and the production of gases (H_2, CO_2, and CH_4).[46]

Inulin is a polydisperse carbohydrate consisting mainly of $\beta(2 \rightarrow 1)$ fructosyl-fructose links, generally referred to as fructan, and is found in various edible fruits and vegetables such as wheat, onions, leeks, garlic, asparagus, artichokes, and bananas.[46,52] Although inulin is not a natural fiber in fish diet, the prebiotic potential of inulin and other dietary fibers may also have interesting applications in aquaculture. Some information is available about fermentation of inulin by fish gut microbiota, notably, *Carnobacterium piscicola*,[4] *C. mobile*,[16] and *Carnobacterium* spp.[8,16] However, it is known that dietary inulin (15% supplement) resulted in damage to intestinal enterocytes of the salmonid fish Arctic charr compared to normal enterocytes from fish fed a 15% supplement of dextrin.[53] On the other hand, more recently it was shown that although the inclusion of dietary inulin did not result in a significant increase in total viable counts, inulin alters the adherent gut microbiota of Arctic charr compared to fish fed dietary dextrin by increasing the proportion of carnobacteria from approximately 5 to 12% of total viable counts (Ringø, Myklebust, and Olsen, unpublished results).

F. Stress

At times of stress, the microbial balance may become disturbed and disordered. Studies undertaken on endothermic animals have reported that chronic stress and social hierarchy formation alter the intestinal microbiota, and the general trend is for the lactobacilli to decrease.[54–58] Several studies have dealt with the effect of stress on gut microbiota in fish.[7,22,59,60] Hierarchy formation affected the total population level of intestinal bacteria colonizing the gut and the microbiota of Arctic charr.[22] *C. divergens*–like isolates were found associated with the large intestine of subordinate fish, while the species were not isolated from the gut of dominant fish.

The digestive tract is a system that reacts to stress-like stimuli, but in fish there is little information available on the effect of handling stress on the intestinal microbiota.[7,59] Ringø and coworkers[7] were not able to show any clear effect on the population levels of viable autochthonous aerobic bacteria or population level of carnobacteria. These results contradict findings in endothermic animals that population levels of LAB in the digestive tract decrease during environmental stress. In the work of Ringø and coworkers[7] it was demonstrated that the numbers of *C. piscicola*–like isolates inhibiting *A. salmonicida* ssp. *salmonicida* was highest (37 of 38) in fish sampled prior to stress, while after 11 days of regular daily handling stress only 16 of 37 *C. piscicola*–like strains inhibit growth of the pathogen. The reason for this difference in the ability to inhibit growth of the fish pathogens in carnobacteria isolates prior to and after stress has not been elucidated and should be a topic of further investigations.

Olsen and coworkers[60] demonstrated using transmission electron microscopy that both prior to excessive handling and 4 hours poststress, numerous bacteria were recovered between enterocyte microvilli of the midgut of Atlantic salmon. The reason why numerous bacteria were found between the microvilli even 4 hours after extensive handling has not been elucidated, but Olsen and coworkers[60] put forward the hypothesis that peel-off of mucus during stress allows bacteria present in the gut lumen to colonize the area between the microvilli. This controversial hypothesis was confirmed by classical microbial analysis that clearly showed that the population level of adherent microbiota decreased in both midgut and hindgut regions with a concomitant increase in feces poststress. In a study of rainbow trout, Ringø and Olsen (unpublished data) confirmed the results of Olsen and coworkers[60] as they demonstrated by scanning electron microscopy relatively high bacterial colonization of hindgut enterocyte surface prior to extensive handling stress, but 4 hours poststress few bacteria were seen at the enterocyte surface. Elimination of the existing microflora including LAB at the enterocyte surface and the lack of protecting mucus in stressed fish might have relevance in pathogenesis. If some of the existing bacteria present in the lumen are pathogenic, it is not unlikely that they can attack the membrane surface and translocate across the intestinal wall, establishing disease.

G. Salinity

It is well known that the intestinal microflora of freshwater and seawater fish harbors different microorganisms.[29,61,62] Some reports have described LAB as a part of the intestinal and gill microbiota in seawater fish.[6,7,21,28,35] Generally, 5–10% of the viable counts of the gut microbiota are lactic acid. In an early study, Strøm[21] found up to 50% lactic acid bacteria in the digestive tract of wild fish, but only 10% in farmed fish. Ringø and coworkers[7] showed that the gut flora of Atlantic salmon consisted of approximately 25% carnobacteria, but in their study a relative low population level ($\sim 2 \times 10^3$ per gram wet tissue) of viable adherent heterotrophic bacteria was associated with the foregut, midgut, and hindgut. A fundamental question that arises when discussing the protective role of the gastrointestinal microbiota is if salinity can affect the antagonistic effect of the gut microbiota. Ringø[63] evaluated the inhibitory effect of carnobacteria colonizing the hindgut of anadromous Arctic charr migrating from freshwater into seawater and in fish migrating from seawater back to freshwater. He was not able to isolate carnobacteria from fish migrating from freshwater into seawater, but eight strains similar to *C. piscicola* were isolated from Arctic charr migrating from seawater back to freshwater. These eight strains did not inhibit *Aeromonas salmonicida* AL 2020, but they did inhibit growth of a *Vibrio* sp, the causative agent of winter ulcer. Salinity and the difference in species must certainly be taken into consideration when discussing antagonism of carnobacteria and other LAB isolated from aquatic animals.

III. USE OF LACTIC ACID BACTERIA IN AQUACULTURE

Specific bacterial pathogens can be an important cause of mortalities in fish hatcheries, as intensive husbandry practices often result in breakdown of the natural host barriers. Research laboratories and commercial hatcheries have attempted to overcome this problem by disinfection of water supplies and food, stimulation of host resistance, and the prophylactic or therapeutic use of antibiotics. However, the indiscriminate use of antibiotics in disease control in many sections of the aquaculture industry has led to selective

pressure of antibiotic resistance in bacteria, a property that may be readily transferred to other bacteria.[64–67] An alternative approach by which opportunistic infections of fish pathogens may be reduced is manipulation of the gut flora either by incorporating antagonistic bacteria into the diet or by dietary manipulation in order to increase the proportion of health-promoting bacteria in the gut microflora. An advantage of these methods is that they can be implemented during the early stages of development when vaccination by injection is impractical. In this regard, the stability of the antagonistic feature is a very important trait of probiotic LAB. According to Olsson,[68] several turbot (*Scophthalmus maximus* L.) and Atlantic salmon LAB isolates lost their capacity to inhibit growth of *Vibrio anguillarum* after being subcultured a limited number of times and stored at −70°C. A similar observation was made by Westerdahl and coworkers,[25] who described that antagonistic activity of several fish intestinal bacteria was rapidly lost after storage and subculturing.

A. Effects of LAB Administration on Intestinal Microbiota

An important criterion for the use of LAB in commercial aquaculture is the colonization potential of LAB in the fish gut, as Vibrionaceae may persist for days or weeks in fish.[69–71] Some studies have suggested that carnobacteria strains are able to survive for several days in the intestine of larval and juvenile fish.[19,23,72,73] Three of these studies[19,72,73] put forward the hypothesis that there is apparently no host specificity with regard to colonization of the fish gut with carnobacteria, unlike in endothermic animals where adhesion of LAB appears to be complicated by host specificity.[74–76] However, additional studies have to be carried out to confirm this controversial hypothesis.

Numerous studies on endothermic animals have demonstrated that administration of LAB affects intestinal microbiota (for review, Refs.[77–80]). However, this effect is less well investigated in fish. Gildberg and Mikkelsen[72] administered two *Carnobacterium divergens* strains, originally isolated from the intestine of mature Atlantic cod and Atlantic salmon, to Atlantic cod juveniles via feed. When the Atlantic cod isolate was used, LAB were only detected in pyloric ceca, while the concentration of LAB was approximately 10-fold higher in the pyloric ceca than in the intestine when the salmon isolate was used.

Transient bacteria may also be efficient if the cells are introduced at high dose. Several investigations conducted during the last two decades have demonstrated that LAB may exert antibacterial effects against undesirable microbes (for review, see Ref.[81]). Based on these results, some investigators have attempted to increase the proportion of LAB associated with the fish digestive tract. In a study with 4-day-old Atlantic cod larvae, Strøm and Ringø[23] used an antagonistic LAB strain, which, when added to the rearing water, favorably influenced the intestinal microbiota of the larvae by increasing the proportion of LAB from approximately 5% to 70% and by a subsequent decrease in the proportion of the bacteria genera *Pseudomonas*, Cytophaga/*Flexibacter*, and *Aeromonas* (Table 3). These results indicate that the LAB were able to colonize and may comprise a major part of the autochthonous microbiota in the gut of the larvae. A similar increase in LAB colonizing the gut was also reported in Atlantic cod fry fed a diet containing *C. divergens*[82] (Table 3). In a study with Atlantic salmon fry, Gildberg and coworkers[83] demonstrated that administration of LAB reported as *Lb. plantarum*, but later reclassified as *C. divergens*,[8] increased the proportion of LAB adherent to intestinal wall from nil to 100% (Table 3).

Table 3 Effect of LAB Administration on Intestinal Microbiota

Fish species	LAB used	Bacterial genera isolated and proportion of microflora population			Refs.
		Before administration (control)	After administration	After challenge	
Atlantic cod—larvae	*C. divergens*	*Pseudomonas* 42.5; Cytophaga/ Flexibacter 42.5; *Aeromonas* 10; *C. divergens* 5	*C. divergens* 70; *Pseudomonas* 20	[e]	[23]
Atlantic cod—fry	*C. divergens*	No information given	No information was given	*C. divergens* 75 *Pseudomonas*-like 25	[82]
Atlantic salmon—fry	*C. divergens*	*Pseudomonas*, Enterobacteriaceae, gram-positive cocci	*C. divergens* 100	*Aer. salmonicida* 90 *C. divergens* 10	[83]
Turbot—larvae	*C. divergens*	*C. divergens* n.d	*C. divergens* (8×10^3)	[e]	[34]
Flounder[a]	*Lactobacillus* sp. DS-12	Enterobacteriaceae 4.3 (5/5); G(+) 4.6 (5/5); yeast 4.6 (5/5); hemolytic bacteria 5.8 (2/5); mucoid colony form 4.8 (1/5); aerobes 8.5 (5/5); anaerobes 7.6 (5/5); aerobes 7.3 (5/5); anaerobes 6.6 (5/5)	Enterobacteriaceae 4.8 (5/5); G(+) 4.3 (5/5); *Lactobacillus* sp. DS-12 7.0 (3/5); *Clostridium* 4.3 (1/5); yeast 4.3 (1/5); hemolytic bacteria 5.1 (1/5)	[e]	[84]
Carp[b]	*S. faecium*	Enterobacteriaceae 6.2; *E. coli* 4.2; *Ent. faecalis* 3.3; *Staph. aureus* 3.7; *Bacillus* spp. 7.0; *Clostridium* spp. 2.9	Enterobacteriaceae 6.2; *E. coli* n.d; *Ent. faecalis* 3.5; *Staph. aureus* 4.0; *Bacillus* spp. 7.0; *Clostridium* spp. 2.7	[e]	[85]
Sheat fish[c]	*Ent. faecium*	*Escherichia coli* 3.1; Enterobacteriaceae 3.0; *Staph. aureus* 4.7 *Bacillus* 6.0; *Clostridium* 2.1	*Escherichia coli* 1.1; Enterobacteriaceae 1.9; *Staph. aureus* 1.4; *Bacillus* 5.6; *Clostridium* n.d	[e]	[86]

(*continued*)

Table 3 Continued

Fish species	LAB used	Bacterial genera isolated and proportion of microflora population			Refs.
		Before administration (control)	After administration	After challenge	
European eel	*Ent. faecium*	*Aeromonas hydrophila* (18)[d]; *Aeromonas sobria* (16); *Chryseobacterium meningosepticum* (3); *Mannheimia haemolytica* (3); *Pasteurella multocida* (12); *Plesiomonas shigelloides* (37); *Pseudomonas aeruginosa* (3); *Pseudomonas stutzeri* (6); *Stenotrophomonas maltophila* (6); *Vibrio* spp. (3); *Vibrio parahaemolyticus* (3)	*Aeromonas caviae* (4); *A. hydrophila* (2); *Aeromonas sobria* (1); *Alcaligenes* spp. (2); *Brevundimonas diminuta* (1); *Burkholderia cepacia* (1); *Ent. faecium* (73); *M. haemolytica* (1); *P. multocida* (2); *P. shigelloides* (4); *P. aeruginosa* (6); *P. stutzeri* (1); *S. maltophila* (1)	[f]	[87]

[a]Data are presented as log 10 and frequency are shown in parentheses.
[b]Data are presented as log 10 after 4 weeks of feeding.
[c]Data are presented as log 10 after 58 days of feeding.
[d]Data presented as percentage.
[e]Challenge test not done.
[f]Challenge test with *Edwardsiella tarda*, but the gut microbiota was not investigated.
n.d., not detected.

In order to increase the proportion of LAB in the digestive tract, Byun and coworkers[84] evaluated the effect of *Lactobacillus* sp. DS-12 administration via the feed on the gut microbiota of flounder (*Paralichthys olivaceus*) after one month of feeding (Table 3). *Lactobacillus* sp. DS-12 was not detected in the intestine of the control group, but 10^7 LAB/g were found in the gastrointestinal tract when the fish were fed the LAB-supplemented feed.

Some studies have evaluated the effect of commercial LAB preparation (*Streptococcus faecium* M74, *Enterococcus faecium* PDFM, and *Ent. faecium* SF68) on the gut microbiota of fish.[85–87] Bogut and coworkers[85] demonstrated that supplementation of 1 g of *S. faecium* M74 per 100 kg feed influenced the intestinal microbiota of 0^+ Israeli carp (*Cyprinus carpio*) to some extent.[85] While *Escherichia coli* disappeared from the intestinal microbiota of the fish after 14 days by feeding the probiotic preparation (Table 3), the population levels of Enterobacteriaceae, *Ent. faecalis*, *Staph. aureus*, *Bacillus* spp., and *Clostridium* spp. were not reduced as a result of including *S. faecium* in the diet.[85] The authors suggested a high adhesive quality in the epithelium of carp digestive tract for *S. faecium.* However, as they only isolated the allochthonous intestinal microbiota, convincing experimental evidence was not provided.

Bogut and coworkers[86] evaluated the effect of *Ent. faecium* PDFM on the intestinal microbiota of Sheat fish (*Silurus glanis*). In this study, the fish were exposed to *Ent. faecium* by including the bacteria in the diet. After approximately 2 months of feeding, some interesting differences in the intestinal microbiota were observed between the two rearing groups. *Ent. faecium* administration decreased the population level of *Staph. aureus*, *E. coli*, and other bacteria of the family Enterobacteriaceae and resulted in complete elimination of *Clostridium* spp. (Table 3).

Recently Chang and Liu[87] investigated the effect of two probiotic bacterial strains, *Ent. faecium* SF68 and *Bacillus toyoi*, on the gut microbiota of European eel. The authors claimed that *Ent. faecium* SF68 began to colonize the intestine on day 4 postinoculation and that the numbers of *Ent. faecium* SF68 reached approximately 2×10^5 on day 14, constituting 73% of the culturable gut microbiota. As a natural consequence of this increase, the population levels of other gut bacteria such as *Aeromonas hydrophila*, *Aeromonas sobria*, *Plesiomonas shigelloides*, *Vibrio* spp., including *Vibrio parahaemolyticus*, were significantly reduced. A detailed description of the intestinal gut microbiota prior to and after supplement of *Ent. faecium* SF68 is shown in Table 3. These results are certainly interesting, but in future studies the autochthonous microbiota, those colonizing the mucus and the area between the intestinal enterocyte microvilli, should be investigated.

When dealing with the potential of probiotics (for example lactic acid bacteria) in aquaculture the fundamental question arises whether it is possible to colonise and maintain the probiotic bacteria within the digestive tract. This is particularly important when long-term exposure may be required for the probiotic effect. In this respect, electron microscope investigations are a useful tool.[88] Figure 1 shows several rod-shaped bacterial profiles associated with microvilli in the hindgut chamber of Atlantic cod. As a pure culture of LAB (*Carnobacterium* ssp., *C. piscicola*, *C. mobile*, and *C. gallinarum*) was isolated in the hindgut chamber,[10] it is suggested that the bacterial profile seen in Figure 1 are LAB. However, this hypothesis must be tested in future studies.

Knowledge about the nutritional contribution of LAB to the production rate of rotifer, *Brachionus plicatilis*, has been evaluated.[89–91] However, control of the microbiota of rotifer cultures has received less attention.

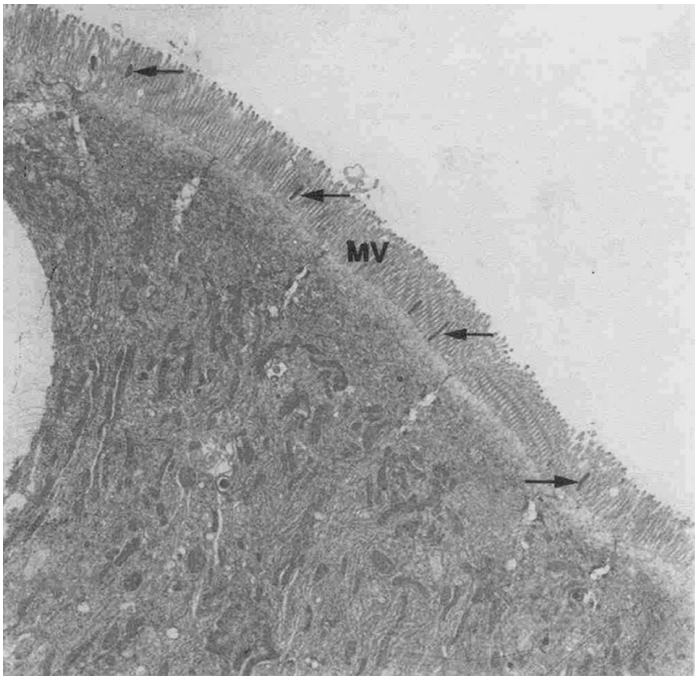

Figure 1 Transmission electron microscopy micrograph of the hindgut chamber of Atlantic cod (*Gadus morhua* L.). Profiles of several bacteria (arrows) are seen between the microvilli (MV). (Adapted from Ref.[10]).

Probiotics, microorganisms, or their products with health benefit to the host have found use in aquaculture as a means of disease control, supplementing or even replacing the use of antimicrobial compounds. A wide range of microalgae (*Tetraselmis*), yeasts (*Debaryomyces*, *Phaffia*, and *Saccharomyces*) and several gram-positive and gram-negative bacteria has been evaluated. Readers with special interest in the prospects of probiotics in aquaculture are referred to recent reviews on this topic.[1,20,92–94]

B. Effects of LAB Administration on Survival and Growth of Fish

During the last decade some research has been conducted on the effect of lactic acid bacteria administration on survival[73,83,95–97] and growth of fish[30,83,85,96,98,101] (Table 4). Garcia de la Banda and coworkers[95] claimed that administration of commercial preparations of live lactic acid bacteria (*Lactococcus lactis* and *Lb. bulgaricus*) via enrichment of rotifer and *Artemia* increased survival of turbot larvae 17 days after hatching. Accelerated growth of Sheat fish after *Ent. faecium* M-74 administration was also reported by Hamácková and coworkers.[96] Ottesen and Olafsen[97] working with Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae demonstrated that *Lb. plantarum* [originally isolated from the adherent gut microbiota of Atlantic cod[21]] administration, 10^6 per mL, to the culture water improved larval survival. At day 12 posthatching (the first critical stage of initial feeding), larval survival was approximately 96% compared to 81.5% survival in the control group (not exposed to LAB). This positive trend in the LAB rearing

Table 4 Effect of LAB Administration on Survival and Growth

LAB isolate used	Host	Way of administration	Effect	Refs.
Survival				
Lac.lactis and *Lb. bulgaricus*	Turbot, larvae	Enrichment of rotifers and *Artemia*	Increased survival of larvae 17 days after hatching	[95]
Ent. faecium M-74	Sheat fish, fry	Addition to diet	Increased survival	[96]
Lb. plantarum[a]	Atlantic halibut, larvae	Addition to culture water	Increased survival of larvae 2 weeks after hatching	[97]
C. divergens[b]	Turbot, larvae	Addition to culture water	No significant effect on larval survival	[34]
Growth				
Str. thermophilus, *Lb. helveticus* or *Lb. plantarum*	Turbot, larvae	Enrichment of rotifer	Enhanced growth	[91]
Lb. coagulans and *S. cerevisiae*	Catla	Addition to diet	Enhanced growth	[99,100]
Lb. acidophilus and *Str. thermophilus*	Nile tilapia, fry	Addition to diet	Enhanced growth	[101]
P. acidilactici	Pollack, larvae	Enrichment of *Artemia*	Enhanced growth	[30]
Ent. faecium M-74	Sheat fish, fry	Addition to diet	Enhanced growth	[96]
Ent. faecium	Israeli carp, adult	Addition to diet	Enhanced growth	[98]
Ent. faecium	Israeli carp, juvenile	Addition to diet	Enhanced growth and feed conversion	[85]
Ent. faecium	Sheat fish, juvenile	Addition to diet	Enhanced growth	[86]
C. divergens[b]	Atlantic salmon, fry	Addition to diet	No significant effect on growth	[83]
Lactobacillus sp. DS-12	Flounder, juvenile	Addition to diet	Varied results	[84]

[a]Isolated from Atlantic cod;[21] reclassified from *Lb. plantarum* to *C. divergens*.[8]
[b]Isolated from Atlantic salmon.[21]

group was also observed later in the rearing period, after 32 days. At this time, larval survival was significantly higher in the rearing group incubated with *Lb. plantarum* (68.4%) compared to the control group (58.2%). Contrary to these results, Ringø[73] did not observe any positive effect on survival of turbot larvae exposed to *C. divergens* (originally isolated from Atlantic salmon) compared to larvae not exposed to lactic acid bacteria (control).

Commercial preparations of *Ent. faecium* seem to improve growth and feed efficiency of Israeli carp[85,98] and Sheat fish.[96] In contrast, Gildberg and coworkers[83] included a *C. divergens* strain (originally isolated from Atlantic salmon) in the diet, but no increased growth of Atlantic salmon fry as a result of LAB administration was observed. In their study on flounder (*Paralichtys olivceus*), the synergistic effect of *Lactobacillus coagulans* and *S. cerevisiae* increased the growth rate of tropical freshwater fish larvae and fry.[99,100] Byun and coworkers[84] checked the feeding effects of *Lactobacillus* sp. DS-12 on fish body weight in two feeding trials. In the first trial, a significant effect of lactic acid bacteria administration seems to be observed. However, the second experiment showed no significant differences between the rearing groups, although there was a tendency to greater increase in body weight as a result of LAB administration.

In a recent study with larval pollack (*Pollachius pollachius*), Gatesoupe[30] concluded that *P. acidilactici* is a promising probiotic, as the best growth results were obtained when *Artemia* had been fed with *P. acidilactici.*

No increase in the growth rate of the rotifers was observed after addition of *Lac. lactis* AR21 through the diet under optimal conditions.[27] Under a suboptimal feeding regime where the food was reduced to 45%, *Lac. lactis* counteracted the growth inhibition of rotifers due to *V. anguillarum* in two of the three experiments performed. However, the authors recovered neither *Lac. lactis* nor *V. anguillarum* from the rotifer after 24 hours.

C. Challenges In Vivo

The major factors involved in the biocontrol of bacterial pathogens in the gastrointestinal tract are primarily those regulating the composition, functions, and interactions of indigenous microbial populations with the animal tissues. This concept is supported by repeated observations that strains of transient enteropathogens can colonize intestinal habitats of endothermic animals. The fact that fish contain intestinal microbiota with antagonistic effects against fish pathogens (for review, see Refs.[88,94]) has prompted investigators to conduct challenge experiments with LAB.[20,27,72,82,83,102,103] However, conflicting mortality results were reported in these studies when comparing the control group with probiotic treatment (Table 5).

Gatesoupe[102] suggested that in vivo experiments with turbot larvae using rotifers grazed on LAB strains (resembling those of *Lb. plantarum* or *Carnobacterium* sp.) improved disease resistance in challenge tests with a pathogenic vibrio (*V. splendidus* strain VS11). However, the results reported in this study were registered after 48 and 72 hours, beyond which the mortality pattern was not discussed. In three later papers, Gildberg and coworkers[72,82,83] used two LAB strains originally isolated from Atlantic salmon and Atlantic cod by Strøm.[21] These two isolates were recently identified by 16S rDNA and AFLP™ fingerprinting as *C. divergens*.[8] In challenge trials with cohabitants with the fish pathogen *A. salmonicida* ssp. *salmonicida*, Gildberg and coworkers,[83] in contrast to expectations, registered the highest mortality of Atlantic salmon fry in fish

Table 5 Challenge Tests of Fish with Lactic Acid Bacteria

LAB isolate used	Host	Pathogen	Way of administration	Effect in challenge test	Suggested mode of action	Refs.
Carnobacterium spp.[a]	Turbot, larvae	*V. splendidus*	Enrichment of rotifers	+	Antagonism and/or improved nutritional value of rotifers	[102]
C. divergens[b]	Atlantic salmon, fry	*A. salmonicida*	Addition to the diet	−		[83]
C. divergens[c]	Atlantic cod, juveniles	*V. anguillarum*	Addition to the diet	+	Not specified	[82]
C. divergens[b]	Atlantic cod, fry	*V. anguillarum*	Addition to the diet	+[e]	Antagonism	[72]
C. divergens[c]	Atlantic cod, fry	*V. anguillarum*	Addition to the diet	−		[72]
Carnobacterium BA211	Rainbow trout	*A. salmonicida*	Addition to the diet	+		[20]
C. inhibens	Rainbow trout	*A. salmonicida*	Addition to the diet	+		[20]
Lb. rhamnosus[d]	Rainbow trout	*A. salmonicida*	Addition to the diet	+		[103]

+, improved disease resistance; −, no significant effect.
[a]Isolated from rotifer.
[b]Isolated from intestine of Atlantic salmon.[21]
[c]Isolated from intestine of Atlantic cod.[21]
[d]A probiotic for human use.
[e]Twelve days after infection significant reduced cumulative mortality was recorded in fish given feed supplemented with *C. divergens* isolated from Atlantic salmon, but no effect was detected 4 weeks after infection.

given the diet containing *C. divergens*, originally isolated from Atlantic salmon intestine. In their study with Atlantic cod fry, Gildberg and Mikkelsen[72] observed the same cumulative mortality whether the *C. divergens* isolates supplemented to the commercial feed were originally isolated from the digestive tract of Atlantic cod or Atlantic salmon when the fish were bath-exposed to *V. anguillarum*. On the other hand, improved disease resistance of Atlantic cod fry was observed when supplementing a commercially dry feed with a strain of *C. divergens* originally isolated from cod.[82] No explanation for these conflicting results has been given. Gildberg and Mikkelsen[72] put forward a hypothesis that bacteriocin production can be inducible and may not occur if the bacteria are not frequently challenged with inhibitors, as previously demonstrated by Schrøder and coworkers.[15]

Recently, Nikoselainen and coworkers[104] investigated the potential probiotic properties of several LAB intended for human or animal use by studying their capacities of adhesion and penetration into fish mucus, their inhibitory effect against fish pathogens and their resistance to fish bile. Furthermore, Nikoselainen and coworkers[103] used the human probiotic *Lb. rhamnosus* (ATCC 53103) in a challenge test with *A. salmonicida* ssp. *salmonicida* with promising results (Table 5). These results should stimulate fish microbiologists to use human probiotic lactic acid bacteria in future studies.

In their study with rainbow trout fingerlings fed *Carnobacterium* BA211 and *C. inhibens* for 14 days, Irianto and Austin[20] showed marked reduction in mortalities compared with controls in a challenge experiment with *A. salmonicida*.

It is well known that rotifers are often suspected of being a vector for bacterial infections to the predating organisms.[105–108] It is therefore surprising that studies dealing with the proliferation of larval pathogens in rotifer cultures are so scarce.[27,91] Gatesoupe[91] reported that the proliferation of *A. salmonicida* that accidentally appeared in the experimental rotifer culture was inhibited by treatment with *Lb. plantarum*. Hazevili and coworkers[27] reported that administration of the probiotic strain *Lac. lactis* AR21 under suboptimal feeding regime counteracted the growth inhibition of the rotifers due to *V. anguillarum*.

IV. ANTAGONISM OF LAB FROM AQUATIC ANIMALS

The ability of LAB to produce antibacterial substances has long been used to preserve foods. Since the days of Metchnikoff, efforts have been made to improve the normal microbiota of the intestine of endothermic animals using LAB.[80,109–112] During the last two decades, numerous experiments have evaluated the ability of LAB (lactobacilli, carnobacteria, enterococci, lactococci, and streptococci) isolated from several fish species and live food (*Brachionus plicatilis*) to inhibit the growth of obligate pathogenic bacteria, and antagonism seems to be common among LAB. Based on these results, Ringø and coworkers[81] suggested that LAB along with other bacteria that belong to the indigenous microbiota of aquatic animals are an important part of the defense mechanism against fish pathogens. Although antagonism is demonstrated under laboratory conditions, such results do not necessarily mean that antagonism is an important mechanism in the natural environment of the microorganisms. The important question to be asked is whether such inhibitory substances result in competitive advantages in the fish gut. Readers with special interest in the antagonistic activity of LAB isolated from aquatic animals are referred to a recent review.[81]

V. PATHOGENIC LACTIC ACID BACTERIA

Lactic acid bacteria have received attention from fishery microbiologists during the last decade because of the increasing interest in their use as potential probiotics and because they are generally considered to be nonpathogenic. However, disease outbreaks caused by LAB have been documented for more than four decades; the involved organisms include *Lactobacillus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Streptococcus*, and *Vagococcus*. Readers with special interest in pathogenic lactic acid bacteria are referred to Ringø and Gatesoupe[2] and Austin and Austin.[113]

VI. CONCLUSIONS AND FUTURE PERSPECTIVES

Numerous investigations have demonstrated that the genera *Lactobacillus*, *Carnobacterium*, *Aerococcus*-like, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Vagococcus*, and *Weissella* belong to the normal microbiota of the gastrointestinal tract in healthy fish. However, it is well known that the population level of lactic acid bacteria associated with the digestive tract is affected by nutritional (chromic oxide, polyunsaturated fatty acids, lipid levels, lipid source, and inulin) and environmental (stress and salinity) factors. Furthermore, the effect of LAB administration on intestinal microbiota, in vivo challenge tests, and the use of commercial preparations of live LAB in aquaculture have been described in several papers.

Further studies on the effect of LAB administration on fish gut microbiota should include molecular approaches to analyze bacterial communities as described for endothermic animals. As conflicting results have been reported in in vivo challenge tests, further studies are needed to clarify whether LAB have a positive effect on fish welfare.

It is generally accepted that the gastrointestinal tract is one of the major infection routes in fish.[88] Based on this fact, one might hypothesize that the autochthonous microbiota associated closely with the intestinal epithelium forms a barrier, serving as the first defense to limit direct attachment to or interactions of pathogenic bacteria with the mucosa. Even though several studies have shown antimicrobial qualities of LAB isolated from different fish species,[81] much work is still needed to clarify if LAB associated closely with the intestinal epithelium form a barrier to limit direct attachment of pathogens in the fish gut.

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22

Future Aspects of Research and Product Development of Lactic Acid Bacteria

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I. INTRODUCTION

Research on lactic acid bacteria (LAB), during recent years has been dominated by advances in molecular biology and the application of the latest genetic and molecular biological tools in both the fundamental and applied areas. These advances have increased our understanding of their taxonomy, metabolism, and interactions with other microbes and, in probiotic applications, ultimately with the host. Especially in the field of probiotics, findings that were only anticipated a few years ago have materialized and more breakthroughs in other areas are to come. The following projections are based on our impressions from the literature, experience, and comparison of the results and claims presented recently.

II. FUNDAMENTAL RESEARCH ASPECTS

The turbulent situation regarding the taxonomy of the actual lactic acid bacteria has stabilized, but within bifidobacteria the situation is still unclear. Undoubtedly much attention

will be focused on this field in the future. Proper taxonomy is a fundamental aspect of the understanding of phylogenetic relationships between species and genera. This, in turn, is of relevance to both the traditional applications of lactic acid bacteria and new potential uses (Fig. 1).

Regarding bifidobacteria and, to some extent, enterococci, one can only hope that the application of new molecular taxonomic criteria gradually calms the field down and the degree of confusion that many researchers now feel changes to new confidence and better understanding and characterization of the organisms studied.

The main break through in the genetics of LAB during recent years has been the advancement of genomics. The total genome sequences of several species are already known, and more will become available. This will undoubtedly enhance our knowledge and understanding of the metabolic potential of LAB, the extent of horizontal gene transfer among them, and their ecological role. The detailed sequence information now available paves the way for subsequent analysis of gene expression at both the transcriptional and translational levels, eventually facilitating the deduction of full metabolic profiles of these organisms in the condition of interest. This information is of relevance also when considering the safety aspects of lactic acid bacteria, i.e., the presence or absence of virulence factors, transmissible antibiotic-resistance genes, or unwanted metabolic activities.

The interaction of LAB with the host microbiota and the physiology, nutrition, and metabolism of higher animals, as well as their role in health and disease, is an area of much speculation. However, new and rigorous studies have started to shed light on this much contested field. Needless to say, understanding these phenomena is a prerequisite for their efficient application.

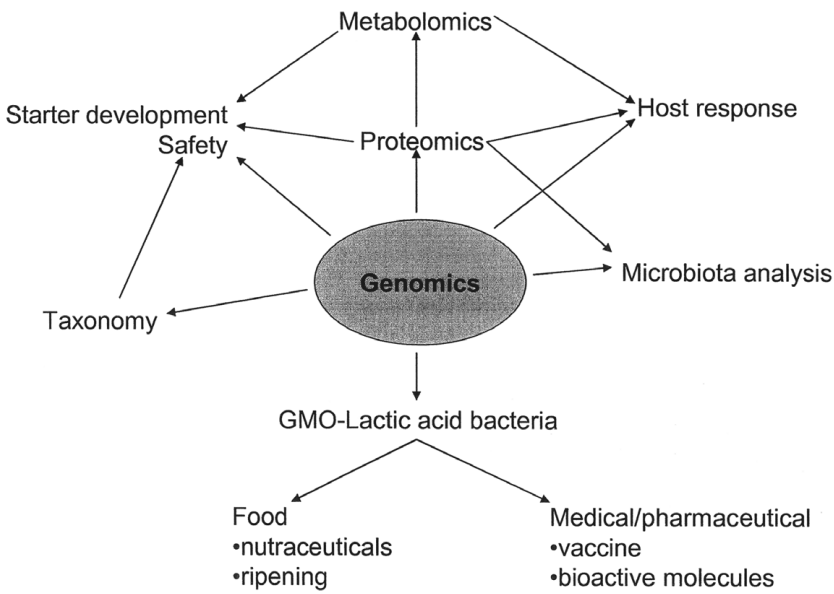

Figure 1 Interaction between different new and emerging technologies for the future investigation of lactic acid bacterium functionality.

III. FUTURE APPLICATIONS

The successes of fundamental research are reflected in the number of applications LAB already have, as well as some totally new industrial uses they may possess. The following list is by no means exhaustive and contains only some of the most obvious ideas that the current state of knowledge allows. It is quite likely that the real breakthroughs are still in the mind of some imaginative inventor outside the common and well-trod paths of research and product development (Fig. 1).

A. Dairy Industry

One of the main technological developments within the dairy industry has been the replacement of traditional starter systems by new starter concentrates and the direct vat set (DVS) concept. This technology requires long-term optimization of each strain to the system, which will be facilitated by the knowledge from the previously described developments in basic research. Despite the advances in dairy technology, bacteriophages remain a significant problem. Better understanding of the molecular biology of bacteriophages and phage-resistance mechanisms together with technology developments will help to eliminate phage-related problems. Although combating phages is a never-ending battle, genetic means of phage control have already shown their value.

Accelerated cheese ripening, control of proteolysis, optimized carbohydrate utilization, and flavor development should also be possible when genetic modification techniques reach the point where they are both ethically and legally acceptable and approved by consumers.

B. Other Industrial Applications

The many kinds of antimicrobial substances produced by several species and strains of lactic acid bacteria could offer alternatives to chemical food additives used to control pathogenic or otherwise harmful contaminants in food. In fermented foods the strains producing antimicrobials could be used themselves, while in other food products isolated and purified antimicrobial substances could be added.

Knowing the genetic basis of the synthesis of bacteriocins opens new possibilities to increase their production on an industrial scale. The success of nisin as a food additive is an encouraging example of the potential in this field. Although new bacteriocins must be evaluated for safety as food additives, the long history of safe human consumption of LAB should make this assessment less costly and laborious than that of purely man-made food chemicals.

With the rapid development of genetic modification of lactic acid bacteria, their potential as production hosts for heterologous proteins in the food and feed industry should also be considered. It should be noted that LAB with new enzymatic activities could have advantages in many of their traditional applications, even though the production of enzymes themselves would not reach levels sufficient to make their production profitable on an industrial scale. As with the dairy applications of genetically modified bacteria, it is of course necessary to ensure that the eventual processes and products are both safe and ethically and legally acceptable.

Advances in the genomic and physiological research of LAB should also improve the selection criteria for strains used in the different processes. As this approach does not involve actual genetic engineering, but is a continuation of traditional technological

product development with modern methods, there should not be any regulatory delays to adapt these techniques.

C. Human and Animal Probiotics

From the studies reviewed in this volume it can be concluded that actual beneficial health effects are associated with certain LAB strains. In addition to prophylactic uses, human therapeutic bacterial preparations may be aimed at special groups, such as patients with pseudomembraneous colitis, viral diarrhea, or recurrent gastrointestinal infections, in whom conventional medication might cause undue stress.

The demonstration of probiotic effects on the level of host microbiota has been greatly enhanced by new molecular biological techniques to detect and identify microbial species and genera that are difficult or impossible to cultivate. Exact identification of probiotic strains in fecal and clinical samples helps to elucidate the extent of bacterial colonization and interaction with the host. With the increased understanding of human molecular biology and recent advances in bioinformatics, it should be possible to study probiotic-host interactions at the level of gene expression in the host.

When probiotic effects have been demonstrated, it can be expected that the underlying mechanisms will be elucidated. This, in turn, will make it possible to design probiotic strains with specific targeted functions. An example of this kind of approach is the *Lactococcus lactis* engineered to produce IL-10.[1] This strain has been effective in suppressing experimental colitis in mice, and investigations on its efficacy in humans have been approved. Other examples with promising results include the use of genetically modified LAB as vehicles for oral vaccines.[2] Because these particular applications are for the treatment and cure of specific diseases, their acceptance by consumers is probably more likely than in the case of genetically modified foods.

Probiotics are also increasingly used as animal feed additives. Antibiotics in animal feeds present risks in the form of transmissible resistance factors endangering human and veterinary chemotherapy, and they are being phased out as zootechnical feed additives in the EU. Thus, there is a need for alternatives, which probiotics could provide. The future of this approach will very much depend on the regulatory developments in the area. The stringency of requirements for quality, efficacy, and especially safety of probiotic preparations will naturally determine the pace with which new products will enter the market. Here, as in the case of human probiotics, the question of acceptable claims should be solved. In the cases of successful animal probiotics, increased productivity is probably often a result of reduced morbidity and mortality. However, claiming these kinds of effects might lead to classification of the product as a veterinary pharmaceutical and hence make it subject to an even more demanding approval procedure.

Whether probiotics are intended for use in humans or animals, it is the responsibility of the regulatory bodies as well the scientific community and marketing companies to ensure that the claims associated with those preparations are as rigorously tested as those of ordinary medicines. The effects are there. What remains is to find the best methods to select beneficial strains or strain combinations as well as to define formulations preserving those properties.

IV. CONCLUSIONS

Lactic acid bacteria have an long history in human cultural traditions. Until recently these organisms have been performing simple and basically similar tasks, differing only in scale

and precision, as they did thousands of years ago. We hope that this volume convinces the reader that new and novel applications based on a better understanding on the potential of lactic acid bacteria in biotechnology, and especially of their role in promoting health and combating disease, are emerging. The potential significance more than justifies multi-disciplinary research in this field, with targets in both food and feed development and promoting human and animal health and well-being.

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