Heping Zhang
Yimin Cai *Editors*

Lactic Acid Bacteria

Fundamentals and Practice

Lactic Acid Bacteria

Heping Zhang · Yimin Cai
Editors

Lactic Acid Bacteria

Fundamentals and Practice

Editors
Heping Zhang
Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education
Inner Mongolia Agricultural University
Hohhot
China

Yimin Cai
Crop, Livestock and Environment Division
Japan International Research Center for Agricultural Sciences
Tsukuba
Japan

ISBN 978-94-017-8840-3 ISBN 978-94-017-8841-0 (eBook)
DOI 10.1007/978-94-017-8841-0
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2014934661

© Springer Science+Business Media Dordrecht 2014
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed. Exempted from this legal reservation are brief excerpts in connection with reviews or scholarly analysis or material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work. Duplication of this publication or parts thereof is permitted only under the provisions of the Copyright Law of the Publisher's location, in its current version, and permission for use must always be obtained from Springer. Permissions for use may be obtained through RightsLink at the Copyright Clearance Center. Violations are liable to prosecution under the respective Copyright Law.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
While the advice and information in this book are believed to be true and accurate at the date of publication, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Contents

1 **Phylogenesis and Evolution of Lactic Acid Bacteria** 1
Zhihong Sun, Jie Yu, Tong Dan, Wenyi Zhang and Heping Zhang

2 **Biodiversity of Lactic Acid Bacteria** 103
Wenjun Liu, Huili Pang, Heping Zhang and Yimin Cai

3 **Genomics of Lactic Acid Bacteria** 205
Wenyi Zhang and Heping Zhang

4 **Proteomics of Lactic Acid Bacteria** 249
Rina Wu and Jing Lu

5 **Lactic Acid Bacteria in Health and Disease** 303
Jinzhong Xiao, Yong Zhang and Zhennai Yang

6 **Lactic Acid Bacteria and the Human Gastrointestinal Tract** 375
Lai-yu Kwok

7 **Application of Lactic Acid Bacteria for Animal Production** 443
Yimin Cai, Huili Pang, Zhongfang Tan, Yanping Wang,
Jianguo Zhang, Chuncheng Xu, Jinsong Yang and Yang Cao

8 **Traditional Chinese Fermented Dairy Foods** 493
Heping Zhang, Xia Chen, Tong Dan and Jie Dong

Contributors

Yimin Cai Crop, Livestock and Environment Division, Japan International Research Centre for Agricultural Sciences, 1-1 Ohwashi, Ibaragi, Tsukuba 305-8686, Japan, e-mail: cai@affrc.go.jp

Yang Cao BaYi Agricultural University, Mishan, China, e-mail: hbdkcaoyang@163.com

Xia Chen Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: chenxia8280@163.com

Tong Dan Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: dantong813218@aliyun.com

Jie Dong Inner Mongolia Normal University, Hohhot, China, e-mail: djnmdongjie@126.com

Lai-yu Kwok Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: kwok_ly@yahoo.com

Wenjun Liu Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: wjliu168@163.com

Jing Lu Chinese Academy of Agricultural Sciences, Institute of Agro-products Processing Science and Technology, Beijing, China, e-mail: ljangela@163.com

Huili Pang Zhengzhou University, Zhengzhou, China, e-mail: pang@zzu.edu.cn

Zhihong Sun Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: sunzhihong78@163.com

Zhongfang Tan Zhengzhou University, Zhengzhou, China, e-mail: tzhongfang@zzu.edu.cn

Yanping Wang Zhengzhou University, Zhengzhou, China, e-mail: wyp@zzu.edu.cn

Rina Wu Shenyang Agricultural University, Shenyang, Liaoning, China, e-mail: wrn6956@163.com

Jinzhong Xiao Food Science and Technology Institute, Morinaga Milk Industry Co. Ltd, Tokyo, Japan, e-mail: laoxiao1212@hotmail.com

Chuncheng Xu China Agriculture University, Beijing, China, e-mail: xucc@cau.edu.cn

Jinsong Yang Hainan University, Haikou, China, e-mail: food868@163.com

Zhennai Yang Beijing Technology and Business University, Beijing, China, e-mail: yangzhennai@th.btbu.edu.cn

Jie Yu Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: yujie8301@sina.com

Heping Zhang Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: hepingdd@vip.sina.com

Jianguo Zhang South China Agricultural University, Guangzhou, China, e-mail: zhangjg@scau.edu.cn

Wenyi Zhang Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot, China, e-mail: zhangwenyizi@163.com

Yong Zhang Research Center for Nutrition and Food Safety, The Third Military Medical University, Chongqing, China, e-mail: zhy-545@163.com

Chapter 1
Phylogenesis and Evolution of Lactic Acid Bacteria

Zhihong Sun, Jie Yu, Tong Dan, Wenyi Zhang and Heping Zhang

Abstract Lactic acid bacteria (LAB) are Gram-positive, usually catalase negative, microaerophilic, acid-tolerant, non-sporulating rods and cocci that reside in a diversity of different habitats. They are widely used in numerous industrial applications, ranging from starter cultures in the dairy industry to probiotics in dietary supplements and bioconversion agents. Despite the functional definition characterising members of the LAB, they are very heterogeneous from a taxonomic viewpoint. Phylogenetic relationships amongst species or subspecies in the LAB have been hotly disputed. Amongst the 'domesticated' bacteria most widely studied and exploited, the LAB are found in two distinct phyla, namely Firmicutes and Actinobacteria. Within the Firmicutes phylum, the most important genera of LAB are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella*, which all belong to the order *Lactobacillales*. Within the Actinobacteria phylum, LAB belong to the *Bifidobacterium* genus. In this chapter, the phylogenetic relatedness and evolutionary history of those eight most important genera of LAB were reviewed.

Z. Sun (✉) · J. Yu · T. Dan · W. Zhang · H. Zhang
Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education,
Inner Mongolia Agricultural University, Hohhot, China
e-mail: sunzhihong78@163.com

J. Yu
e-mail: yujie8301@sina.com

T. Dan
e-mail: dantong813218@aliyun.com

W. Zhang
e-mail: zhangwenyizi@163.com

H. Zhang
e-mail: hepingdd@vip.sina.com

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_1,

© Springer Science+Business Media Dordrecht 2014

Keywords Lactic acid bacteria · Taxonomy · Phylogenetic relatedness · Evolutionary

1.1 Introduction

The term 'lactic acid bacteria' does not relate to a phylogenetic class of organisms, but rather to the metabolic capabilities of the species whining this group. Lactic acid bacteria (LAB) are historically defined as a ubiquitous and heterogeneous family of microbes that can ferment various nutrients into, primarily, lactic acid. LAB are Gram-positive, usually catalase negative, microaerophilic, acid-tolerant, non-sporulating rods and cocci that reside in a diversity of different habitats. These include human cavities such as the gastrointestinal tract, oral cavity, respiratory tract and vaginal cavity, as well as a number of environmental niches such as plants and processed dairy, meat and vegetable products (Klaenhammer et al. 2002, 2005; Kleerebezem and Hugenholtz 2003). LAB are widely used in numerous industrial applications, ranging from starter cultures in the dairy industry to probiotics in dietary supplements and bioconversion agents. Amongst the 'domesticated' bacteria most widely studied and exploited, the LAB are found in two distinct phyla, namely Firmicutes and Actinobacteria. Within the Firmicutes phylum, the most important genera of LAB are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella*, which all belong to the order *Lactobacillales* and are low-GC content organisms (31–49 %). Within the Actinobacteria phylum, LAB belong to the *Bifidobacterium* genus, which have a high-GC content (58–61 %) (Klaenhammer et al. 2005; Pfeiler and Klaenhammer 2007; Schleifer and Ludwig 1995a; Horvath et al. 2009).

LAB play an important role in many industrial fermentation processes and human nutrition. Due to their presence in the gastrointestinal tract, some members have emerged as probiotics since they are of human origin and confer benefits on human health (Klaenhammer et al. 2008; Makarova et al. 2006). Despite the functional definition characterising members of the LAB, they are very heterogeneous from a taxonomic viewpoint (Hammes and Vogel 1995; Zhang et al. 2011; Salvetti et al. 2013). Phylogenetic relationships amongst species in the LAB have been hotly disputed. Based on the phylogenetic relatedness of 16S ribosomal ribonucleic acid (16S rRNA) sequences from different species (Woese 1987), LAB have been divided into two major branches, the *Clostridium* branch and the actinomycetes branch. The typical LAB, such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus* and *Pediococcus*, belong to the *Clostridium* branch. In contrast, the genus *Bifidobacterium* belongs to the actinomycetes branch (Schleifer and Ludwig 1995a; Stiles and Holzapfel 1997). One of the foremost debates in LAB phylogeny concerns species in the gener a *Lactobacillus*, *Pediococcus* and *Leuconostoc*, which belong to the families *Lactobacillaceae* and *Leuconostocaceae*, these debates have arisen due to severe disagreements concerning the types of analyses applied to the different available

datasets (Pfeiler and Klaenhammer 2007; Makarova et al. 2006; Collins et al. 1991; Carr et al. 2002; Cai et al. 2009; Claesson et al. 2008). Recently, a number of LAB genomes have been sequenced and the subsequent explosion of genomic information has facilitated a better understanding of LAB characteristics, such as their physiology, metabolic capabilities, key gene features and niche adaptation. Moreover, the availability of genome sequences has provided a good opportunity to understand LAB phylogenetic relatedness and evolutionary history (Klaenhammer et al. 2008).

1.2 The Genus *Bifidobacterium*

1.2.1 History

The first isolation of a *Bifidobacterium* species was from the faeces of breast-fed infants in 1899, by Henri Tissier, and at the time, was designated as *Bacillus bifidus* (Tissier 1899, 1900). Even though Orla-Jensen proposed the genus *Bifidobacterium* in 1924 (le Dr ORLA-JENSEN 1924), these species continued to be classified into other taxonomic groups for several decades; for example *Bacillus bifidus*, *Bacteroides bifidus* from 1923 to 1934 and *Lactobacillus bifidus* from 1939 to 1957 (Sgorbati et al. 1995; Biavati and Mattarelli 1924, 2006). In the 1970s, these species were reclassified as a separate taxon and designated as the genus *Bifidobacterium* comprising 11 species (Poupard et al. 1973; Bergey et al. 1974). This was updated to 24 species in 1986 (Scardovi 1986) and then 32 species and 9 subspecies in 2012 (Biavati et al. 2012). Currently, there are 41 proposed species and 9 subspecies (http://www.bacterio.net/bifidobacterium.html, 20 December 2013). These have been isolated from the gastrointestinal tract (GI) of humans, animals and insects, and also from human dental caries and dairy products (Table 1.1).

DNA G+C content (mol.%): 46–67.
Type species: *Bifidobacterium bifidum* (Tissier 1900) Orla-Jensen 1924.

1.2.2 Cell Morphology and Cultural Characteristics

Bifidobacterium species are nonmotile, nonsporeforming, non-gas producing, Gram-positive, catalase-negative bacteria with a high GC content (46–67 %) (Biavati and Mattarelli 1924, 2006). Their morphology is generally referred to as bifid or irregular V- or Y-shaped rods resembling branches. The actual reason for the irregular shape of bifidobacteria is not yet clearly understood. However, a few studies have revealed that absence or low concentrations of N-acetylamino sugar (Glick et al. 1960), Ca^{2+}ions (Kojima et al. 1970) or amino acids (Husain et al. 1972) in the growth media can induce the bifid shape.

Table 1.1 List of the species in the genus *Bifidobacterium*

Number	Species	Isolated date	Source classification	Phylogroup*
1	*B. actinocoloniiforme*	2011	Bee-GI	B. asteroides
2	*B. adolescentis*	1963	Human-GI	B. adolescentis
3	*B. angulatum*	1974	Human-GI	B. adolescentis
4	*B. animalis* ssp. *animalis*	2004	Rodent-GI	B. pseudolongum
5	*B. animalis* ssp. *lactis*	1997	Dairy products	B. pseudolongum
6	*B. asteroides*	1969	Bee-GI	B. asteroides
7	*B. biavatii*	2012	Monkey-GI	B. scardovii
8	*B. bifidum*	1924	Human-GI	ss
9	*B. bohemicum*	2011	Bee-GI	ss
10	*B. bombi*	2009	Bee-GI	ss
11	*B. boum*	1979	Bovine-GI	B. boum
12	*B. breve*	1963	Human-GI	ss
13	*B. callitrichos*	2012	Monkey-GI	B. adolescentis
14	*B. catenulatum*	1974	Human-GI	B. adolescentis
15	*B. choerinum*	1979	Swine-GI	B. pseudolongum
16	*B. coryneforme*	1982	Bee-GI	B. asteroides
17	*B. cuniculi*	1979	Rodent-GI	B. cuniculi
18	*B. dentium*	1974	Human-dental	B. adolescentis
19	*B. gallicum*	1990	Human-GI	B. pseudolongum
20	*B. gallinarum*	1983	Poultry-GI	B. pullorum
21	*B. indicum*	1969	Bee-GI	B. asteroides
22	*B. kashiwanohense*	2011	Human-GI	B. adolescentis
23	*B. longum* ssp. *infantis*	2008	Human-GI	B. longum
24	*B. longum* ssp. *longum*	1963	Human-GI	B. longum
25	*B. longum* ssp. *suis*	2008	Swine-GI	B. longum
26	*B. magnum*	1974	Rodent-GI	ss
27	*B. merycicum*	1991	Bovine-GI	B. adolescentis
28	*B. minimum*	1982	Sewage	ss
29	*B. mongoliense*	2009	Dairy products	ss
30	*B. pseudocatenulatum*	1979	Human-GI	B. adolescentis
31	*B. pseudolongum* ssp. *globosum*	1992	Bovine-GI	B. pseudolongum
32	*B. pseudolongum* ssp. *pseudolongum*	1992	Swine-GI	B. pseudolongum
33	*B. psychraerophilum*	2004	Swine-GI	B. cuniculi
34	*B. pullorum*	1974	Poultry-GI	B. pullorum
35	*B. reuteri*	2012	Monkey-GI	ss
36	*B. ruminantium*	1991	Bovine-GI	B. adolescentis
37	*B. saeculare*	1992	Rodent-GI	B. pullorum
38	*B. saguini*	2012	Monkey-GI	ss
39	*B. scardovii*	2002	Human-blood	B. scardovii
40	*B. stellenboschense*	2012	Monkey-GI	B. scardovii
41	*B. stercoris*	2010	Human-GI	B. adolescentis
42	*B. subtile*	1982	Sewage	ss

(continued)

Table 1.1 (continued)

Number	Species	Isolated date	Source classification	Phylogroup*
43	*B. thermacidophilum* ssp. *porcinum*	2003	Swine-GI	B. boum
44	*B. thermacidophilum* ssp. *thermacidophilum*	2003	Sewage	B. boum
45	*B. thermophilum*	1969	Swine-GI	B. boum
46	*B. tsurumiense*	2008	Rodent-dental	ss

* ss, single species

The majority of species are obligate anaerobes, but some species, such as *B. psychraerophilum*, *B. scardovii* and *B. tsurumiense*, can tolerate O_2 and grow under aerobic conditions (Okamoto et al. 2008). The optimum growth temperature is 37–41 °C for most species with minimum and maximum growth temperatures of 25 and 45 °C, respectively. It is worth mentioning that *B. psychraerophilum* can grow at 8 °C and *B. thermacidophilum* can grow at 49.5 °C (Biavati and Mattarelli 1924, 2006). The optimum pH for initial growth is 6.5–7.0. With the exception of *B. thermacidophilum*, other species in the genus cannot grow at pH 4.5–5.0 or pH 8.0–8.5.

1.2.3 Phylogenetic Position of Species in the Genus **Bifidobacterium**

The genus of *Bifidobacterium* belongs to the family *Bifidobacteriaceae*, order *Bifidobacteriales*, phylum Actinobacteria and is the type genus for the family *Bifidobacteriaceae* (Biavati and Mattarelli 1924, 2006). The phylogenetic position of *Bifidobacterium* was established using 16S rRNA gene sequence analysis (Schleifer and Ludwig 1995a). Phylogenetic relationships analysis indicated that the genus *Bifidobacterium*, even if traditionally listed among LAB, is only poorly phylogenetically related to other LAB. Furthermore, all species in the genus *Bifidobacterium* use a particular metabolic pathway (the bifid shunt) for degradation of hexoses which is different to the other genera of LAB including *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* (Felis and Dellaglio 2007).

The size of bifidobacteria genome ranges from 1.9 to 2.8 Mb (Lee and O'Sullivan 2010). By now, 54 genomes from 14 different species and subspecies were published. These species and subspecies include *B. adolescentis*, *B. angulatum*, *B. animalis* subsp. *lactis*, *B. animalis* subsp. *animalis*, *B. asteroides*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. gallicum*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. pseudocatenulatum* and *B. thermophilum*.

1.2.4 Grouping Within the Genus **Bifidobacterium**

Traditionally, species of *Bifidobacterium* have been identified on the basis of the location or host from which they were isolated, example for, *B. animalis*, *B. adolescentis* and *B. dentium*, their cell morphology, from analysis of fermentation products and associated enzyme activities, or by their ability to utilise various sugar substrates (Ventura et al. 2004a). Unfortunately, all these phenotypic methods suffer from a certain lack of reproducibility as they vary depending on the culture conditions used in different laboratories and between isolates from the same species. During the last decade, the development of molecular biological tools has led to profound modifications in the identification methodologies for these bacteria and has resulted in various adjustments in their classification. Genotypic identification methods using the 16S and 23S rDNA genes are more accurate, although they cannot clearly differentiate between all *Bifidobacterium* species, for example, *B. catenulatum* and *B. pseudocatenulatum*, *B. adolescentis* and *B. stercoris*, *B. coryneforme* and *B. indicum*, showed more than 99 % homology.

A phylogenetic analysis based on the 16S rRNA gene sequences of the type strains of the 46 currently recognised species and subspecies of the genus *Bifidobacterium* resulted in a phylogenetic tree (Fig. 1.1) that was consistent with previously described bifidobacterial taxonomic analyses (Felis and Dellaglio 2007; Ventura et al. 2004a; Miyake et al. 1998). The 16S rRNA gene allowed the discrimination of most species within the genus *Bifidobacterium*. Phylogenetic analysis of 16S rRNA gene sequences showed incongruence between the ecological origin of species and their evolutionary groupings. According to their phylogenetic relationships, the 46 validated species and subspecies of the genus *Bifidobacterium* could be divided into 8 phylogenetic groups and 11 single species (Fig. 1.1).

The *Bifidobacterium adolescentis* group comprises ten species, *B. adolescentis*, *B. angulatum*, *B. callitrichos*, *B. catenulatum*, *B. dentium*, *B. kashiwanohense*, *B. merycicum*, *B. pseudocatenulatum*, *B. ruminantium* and *B. stercoris* (Fig. 1.1). Three of them, *B. callitrichos*, *B. kashiwanohense* and *B. stercoris*, were added with respect to Felis and Dellaglio (Felis and Dellaglio 2007). All strains of the type species are isolated from faeces of human and animal (Scardovi and Crociani 1974; Endo et al. 2012; Morita et al. 2011a; Biavati and Mattarelli 1991; Scardovi et al. 1979; Kim et al. 2010). The *B. asteroides* group contains four species, *B. actinocoloniiforme*, *B. asteroides*, *B. coryneforme* and *B. indicum* (Fig. 1.1). One new species, *B. actinocoloniiforme*, was added in 2011 (Killer et al. 2011). Interestingly, the type strains of all species in the *B. asteroides* group were isolated from bumblebee GIs (Killer et al. 2011; Scardovi and Trovatelli 1969; Biavati et al. 1982). In this group, the phylogenetic relationship amongst *B. indicum*, *B. coryneforme* and *B. asteroides* was very close. Based on 16S rRNA gene sequences, *B. indicum* and *B. asteroides* could be distinguished, but *B. indicum* and *B. coryneforme* could not. Subsequently, Ventura et al. (2006) were successful in separating *B. indicum* and *B. coryneforme*, even though they were very closely related.

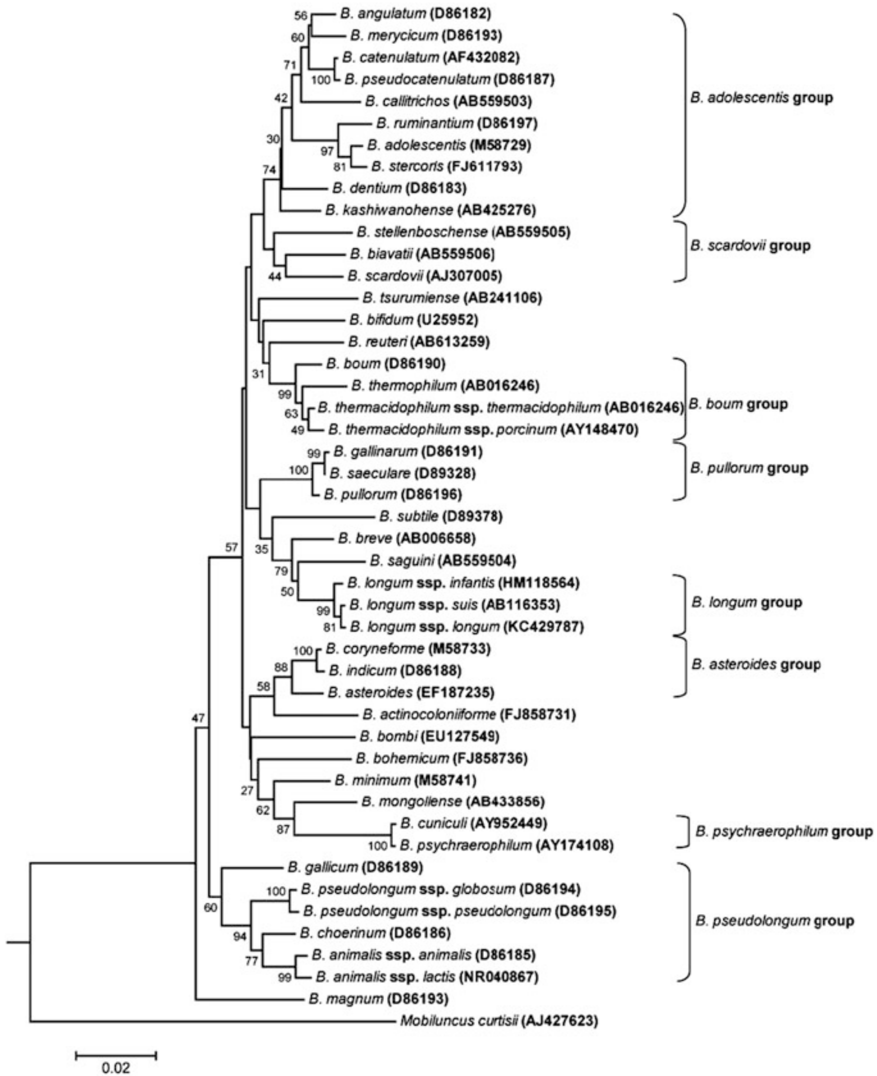


Fig. 1.1 Phylogenetic tree depicting the relationships between *Bifidobacterium* species based on 16S rRNA gene sequences. The tree was constructed through the neighbour-joining method with 1000 bootstrap replicates

The *B. boum* group comprises four species and subspecies, *B. boum*, *B. thermacidophilum* subsp. *porcinum*, *B. thermacidophilum* subsp. *thermacidophilum* and *B. thermophilum* (Fig. 1.1). The *B. pseudolongum* group is constituted of six species and subspecies, *B. animalis* subsp. *animalis*, *B. animalis* subsp. *lactis*, *B. choerinum*, *B. gallicum*, *B. pseudolongum* subsp. *globosum* and *B. pseudolongum* subsp. *pseudolongum* (Fig. 1.1). As in the *B. adolescentis* group, the main

type isolates in the *B. pseudolongum* group was isolated from human and animal GIs (Scardovi et al. 1979; Lauer 1990; Yaeshima et al. 1992), except for *B. animalis* subsp. *lactis* which was isolated from yogurt (Masco et al. 2004). The *B. pullorum* group comprises three species, *B. gallinarum*, *B. pullorum* and *B. saeculare* (Fig. 1.1). The *B. longum* group comprises three subspecies of *B. longum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum* and *B. longum* subsp. *suis* (Fig. 1.1). These four groups are all consistent with previous descriptions (Felis and Dellaglio 2007). The most noteworthy changes with respect to previous surveys are in the *B. scardovii* group (*B. biavatii*, *B. scardovii* and *B. stellenboschense*) and the *B. cuniculi* pair (*B. cuniculi* and *B. psychraerophilum*) due to the descriptions of new species (*B. biavatii* and *B. stellenboschense*) (Endo et al. 2012).

1.2.5 Phylogeny and Evolution

To accurately identify the species or subspecies within the genus *Bifidobacterium*, a variety of molecular techniques based on 16S rRNA gene sequence analysis have been used, including amplified rDNA restriction analysis (ARDRA) (Ventura et al. 2001a; Venema and Maathuis 2003) and species-specific PCR primers (Matsuki et al. 1999, 2002; Dong et al. 2000; Ventura et al. 2001b; Ventura and Zink 2002).

In 2001, Ventura et al. (2001a) developed an ARDRA method based on the 16S rDNA sequences which allowed discrimination of 14 out of 16 *Bifidobacterium* species. This study demonstrated that species-specific detection of *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. coryneforme*, *B. cuniculi*, *B. dentium*, *B. infantis*, *B. magnum*, *B. pseudocatenulatum* and *B. pullorum* present in different micro-ecological environments could be accomplished in a reliable, rapid and accurate manner. However, the closely related subsp. *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* could not be distinguished from each other using this method and neither could the two subsp *B. longum* subsp. *longum* and *B. longum* subsp. *suis*. Subsequently, Venema and Maathuis (2003) developed other ARDRA protocols for further identification of *Bifidobacterium* species. Unfortunately, the large number of restriction enzymes involved makes this protocol labour-intensive, time-consuming, and consequently, a low-throughput method preventing screening of large numbers of isolates (Ventura et al. 2004a).

To cover all bifidobacterial species that had been isolated and identified from the human GI, Matsuki research team (Matsuki et al. 1999) developed species-specific primers targeting the 16S rDNA sequences of *B. longum*, *B. infantis*, *B. dentium*, *B. gallicum*, *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum* and *B. pseudocatenulatum*. However, a limitation of species-specific primers is that large-scale studies, and repeated PCR amplification cycles with different set of primers are required to analyse species composition. To avoid this inconvenience, Dong et al. (2000) developed a multiple PCR primers strategy for the detection of *B. bifidum*, *B. adolescentis*, *B. infantis*, *B. longum* and *B. breve*. In

fact, a mixture of the five species-specific primers that target different sites of the 16S rDNA were used simultaneously to identify all five species in individual or mixed cultures.

During the last two decades, the advent of molecular tools based on DNA fingerprinting has greatly enhanced approaches for detection, differentiation and identification of *Bifidobacterium* species, particularly the use of randomly amplified polymorphic DNA (RAPD) (Vincent et al. 1998), restriction fragment length polymorphism (RFLP) (Roy and Sirois 2000), enterobacterial repetitive intergenic consensus sequences (ERIC)-PCR (Ventura et al. 2003b), pulsed field gel electrophoresis (PFGE) (Roy et al. 1996) and amplified fragment length polymorphism (AFLP) (Dimitrov 2011; Makino et al. 2011). Many of these methods are cultivation-independent allowing in situ analysis, thus removing the limitations of culturing. Furthermore, they have the capacity for high resolution and provide reproducible data, making them suitable for both species identification and strain typing. However, these molecular techniques are limited by how extensive any particular fingerprint database is. Consequently, as the contents of the various databases for these different techniques expand, this approach will improve in overall usefulness.

In recent years, systematic evaluation of evolutionary relatedness has been achieved using alternative molecular markers d for phylogenetic analysis of *Bifidobacterium* species, including *gro*EL (Jian et al. 2001; Ventura and Zink 2003; Ventura et al. 2004b), *gro*ES (Ventura et al. 2004b), *rec*A (Ventura and Zink 2003; Kullen et al. 1997), *tuf* (Ventura and Zink 2003), *atp*D (Ventura et al. 2004a), *dna*K (Ventura et al. 2005) and *grp*E (Ventura et al. 2005). However, there are currently no complete sequence databases available for such genes and these molecules do not fulfil all the criteria in order to be considered as suitable evolutionary clocks. Molecular methods using a single gene or two genes are popular because they yield quick and unequivocal results, but in some cases suffer from the disadvantage that they are not sufficiently discriminatory (Ventura et al. 2006). Consequently, the combined use of several genes for detailed classification purposes, also called multilocus sequence typing (MLST) approach, recommended by ad hoc committee for the re-evaluation of the bacterial species (Stackebrandt et al. 2002). It recommends that, for the description of novel species, sequences from four or five housekeeping genes besides the 16S rRNA gene should be taken into account.

Ventura et al. (2006) developed a MLST scheme based on seven housekeeping genes, *clp*C, *dna*B, *dna*G, *dna*J1, *pur*F, *rpo*C and *xfp*, to assess type isolates of *Bifidobacterium* species. In this study, the phylogenetic tree resulting from the concatenated sequences of 31 type strains of species in the genus *Bifidobacterium* was presented. Each species was differentiated as a distinct entity. The mean similarity of these concatenated sequences was 78.26 % compared with 93.85 % using the 16S rRNA gene alone and the distance matrix included a smaller number of low values. The results demonstrated that the concatenation of these seven gene sequences for phylogenetic purposes allowed a significant increase in the discriminatory power between taxa. Furthermore, it was revealed that all bifidobacterial

phylogenetic groups were derived from a single ancestor in the *B. asteroides* group. In contrast, speciation of this remnant taxa had evolved more recently.

Subsequently, to determine the genetic diversity and population structure of *Bifidobacterium* species, Deletoile et al. (2010) reported a species delineation and clonal diversity in four *Bifidobacterium* species based on MLST of seven house-keeping genes. Through phylogenetic analysis of concatenated sequences, 119 *Bifidobacterium* isolates from *B. animalis*, *B. bifidum*, *B. breve* and *B. longum*, clustered in groups that corresponded to previously named taxa, and suggested that *B. longum* subsp. *infantis* was a nascent lineage emerging from within *B. longum* subsp. l*ongum*. Simultaneously, microevolutionary analysis demonstrated frequent homologous recombination in these species indicating that clonal diversification had been accelerated which may facilitate the transfer of biological properties amongst species.

To verify the mother-to-infant transmission of *B. longum* subsp. *longum*, Makino et al. reported that 207 isolates from 8 pairs of mothers and infants could be discriminated by MLST and AFLP analysis. In their study, 11 strains of *B. longum* subsp. *longum* were found to be monophyletic for the faeces of the mother and her infant, and were found in the first faeces of the infant and in the faeces at days 3, 7, 30 and 90 after birth. The strains isolated from each family did not belong to clusters derived from any of the other families, suggesting that each mother–infant pair might have unique family specific strains. This finding confirms that these strains were transferred from the intestine of the mother to that of the infant.

Recently, the increasing number of available bacterial genome sequences has provided a large number of housekeeping gene sequences that can be used both for species identification, for understanding evolution and to improve our knowledge of the diversity and evolution of commensal bacteria of the GIT. In 2010, Bottacini et al. (2010) reported genome-level phylogenetic investigation. Although sequencing only a few genomes from a limited number of species was insufficient to understand the overall genomic basis and plasticity of the *Bifidobacterium* genus, a supertree was constructed using a core gene set comprising 506 orthologues. This tree was largely concordant with the phylogenetic tree obtained using 16S rRNA genes. However, the discriminatory power of the concatenated tree was much higher than that observed using the single 16S rRNA gene, which was also confirmed by analysis of the pairwise distances and the standard deviation, respectively.

1.3 The Genus *Enterococcus*

1.3.1 History

The word 'enterocoque' or 'enterococci' was first used by Thiercelin and Jouhaud in 1899 (Thiercelin and Jouhaud 1903) to describe a new Gram-positive diplo-coccal bacterium of intestinal origin that would later be included in the genus

Enterococcus as the type isolate of the species *Enterococcus proteiformis*. Andrewes and Horder later renamed Thiercelin's 'enterocoque' as *Streptococcus faecalis* based on its ability to form short or long chains. These associations between *Enterococcus* and *Streptococcus* mean that they cannot be considered separately, especially in early studies. However, Lancefield developed a serological typing system for both genera in which those of faecal origin could be distinguished because they possessed a group D antigen (Lancefield 1933). This correlated with the groupings of Sherman (Sherman 1937), who proposed a new classification scheme for the genus *Streptococcus* that divided the streptococci into four groups designated pyogenic, viridans, lactic and enterococcal. The enterococcal, or group D group, included *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus bovis* and *Streptococcus equines*.

The use of DNA hybridisation and 16S rRNA sequencing in 1984 established that the species *S. faecium* and *S. faecalis* were sufficiently distinct from other *Streptococcus* species to be designated as a new genus: *Enterococcus* (Foulquie et al. 2006). This meant that the D group (*Enterococcus* group) antigen could be found in both *Streptococcus* and *Enterococcus* species. Subsequently nine more species have been transferred from the *Streptococcus* genus and currently the genus *Enterococcus* includes 46 validated species one of which is comprised of two subspecies: *E. alcedinis*, *E. aquimarinus*, *E. asini*, *E. avium*, *E. caccae*, *E. camelliae*, *E. canintestini*, *E. canis*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. devriesei*, *E. dispar*, *E. durans*, *E. eurekensis*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. gilvus*, *E. haemoperoxidus*, *E. hermanniensis*, *E. hirae*, *E. italicus*, *E. lactis*, *E. lemanii*, *E. malodoratus*, *E. moraviensis*, *E. mundtii*, *E. ococcusvillorum*, *E. pallens*, *E. phoeniculicola*, *E. plantarum*, *E. pseudoavium*, *E. quebecensis*, *E. raffinosus*, *E. ratti*, *E. rivorum*, *E. rotai*, *E. saccharolyticus* subsp. *saccharolyticus*, *E. saccharolyticus* subsp. *taiwanensis*, *E. silesiacus*, *E. sulfurous*, *E. termitis*, *E. thailandicus*, *E. ureasiticus*, *E. ureilyticus* and *E. viikkiensis* (http://www.bacterio.net/enterococcus.html, 20 December 2013).

DNA G+C content (mol.%): 35.1–44.9.
Type species: *Enterococcus faecalis* (Andrewes and Horder 1906) Kilpper-Bälz and Schleifer (1984).

1.3.2 Cell Morphology and Cultural Characteristics

No phenotypic criteria are available by which the genus *Enterococcus* can be separated unequivocally from other genera. However, species within the genus have common attributes. The cells are ovoid, Gram-positive and occur singly, in pairs or in short chains. Within the chains the cells are frequently arranged in pairs and elongated in the direction of the chain. The classical species *E. faecalis* and *E. faecium* on which the genus description (Schleifer et al. 1984) was based, have a number of characteristics in common that distinguish them from other catalase

negative, Gram-positive, facultatively anaerobic cocci with which they could be confused: their ability to grow both at 10 mand 45 °C, in 6.5 % NaCl broth and at pH 9.6 (Devriese et al. 1987; Pavel et al. 1984). However, other species, added later to the genus, do not show all of these characteristics.

In cases where only presence/absence of the genus *Enterococcus* in a sample is required, it is only possible to determine by identification of the component species. This means that genus identification necessarily follows species identification: e.g. when an isolate shows characteristics of one of the species described below, the isolate is an *Enterococcus.* For practical reasons if an isolate has the following attributes it can be presumed to belong to the genus *Enterococcus*: catalase negative, Gram-positive cocci, able to grow in 6.5 % (w/v) NaCl broth, showing good growth on media containing 0.04 % sodium azide (commonly used in selective isolation of *Enterococcus* species).

1.3.3 Phylogenetic Position and Genomes of Species in the Enterococcus genus

The genus *Enterococcus* belongs to the family *Enterococcaceae*, order *Lactobacillales*, phylum Firmicutes and is the type genus of *Enterococcaceae* family (Pavel et al. 1984). The phylogenetic position of the genus *Enterococcus* was established using 16S rRNA gene sequence analysis by Schleifer and Ludwig (1995a) and phylogenetic relationship analysis showed it to be closely related to the genera *Lactococcus* and *Streptococcus.*

The size range of complete *Enterococcus* genomes ranged from 1.74 to 2.76 Mb: the size of two type strains of *Enterococcus* ranged from 2.0 to 2.28 Mb (Jayarao et al. 1992). To date, more than 200 genomes from isolates of *Enterococcus* species have been sequenced and are available including the sequences of *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. italicus*, *E. mundtii* and *E. saccharolyticus*.

1.3.4 Taxonomy and Phylogenetics

Identification of *Enterococcus* species has always been problematic using physiological tests, because many species vary by only one phenotypic trait (Knudtson and Hartman 1992; Koort et al. 2004; Miranda et al. 1992; Park et al. 1999). Furthermore, identification of species by conventional tests often requires long incubation times (Facklam et al. 2002). Genotypic identification methods using the 16S and 23S rDNA genes are more accurate, although they cannot differentiate between all *Enterococcus* species, for example, *E. devriesei*, *E. viikkiensis* and *E. pesudoavium* showed 100 % homology; *E. gallinarum* and *E. casseliflavus*

showed 99.8 % homology. Despite these close relationships and similarities the species are well separated by DNA–DNA similarity determinations and other molecular identification techniques. The 46 validated species in the genus *Enterococcus* can be divided into three phylogenetic groups, six pairs (groups containing only two species) and singletons based on their 16S rRNA gene sequence (Fig. 1.2).

1.3.4.1 *Enterococcus faecalis* Group

The *E. faecalis* group comprises 12 species, *E. caccae*, *E. silesiacus*, *E. ureasiticus*, *E. rotai*, *E. ureilyticus*, *E. plantarum*, *E. moraviensis*, *E. haemoperoxidus*, *E. quebecensis*, *E. termitis*, *E. faecalis* and *E. rivorum* (Fig. 1.2). Although phylogenetic distances between species are large in the *E. faecalis* group, the 12 species share many phenotypic traits. They form similar dark red colonies with a metallic sheen on Slanetz–Bartley agar can grow at 10 °C, in 6.5 % NaCl and are D group antigen positive (Pavel et al. 1984; Devriese et al. 2006; Devriese and Pot 1995). Nevertheless, these species can be differentiated from each other using a series of biochemical tests.

1.3.4.2 *Enterococcus faecium* Group

The *E. faecalis* group comprises eight species, *E. canis*, *E. mundtii*, *E. durans*, *E. thailandicus*, *E. hirae*, *E. ratti*, *E. faecium* and *E. lactis* (Fig. 1.2). These species generally have identical growth and physiological characteristics. Discrimination between individual species in the *E. faecalis* group is often unreliable using biochemical tests (Pavel et al. 1984). *E. durans*, *E. thailandicus* and *E. hirae*, are especially difficult to differentiate, although they can be clearly distinguished by whole-cell protein profile analysis using SDS-PAGE, tDNA-PCR and arbirtrarily primed PCR analysis (Devriese et al. 2002).

1.3.4.3 *Enterococcus avium* Group

The *E. avium* group comprises seven species, *E. devriesei*, *E. viikkiensis*, *E. pseudoavium*, *E. avium*, *E. raffinosus*, *E. gilvus* and *E. malodoratus*. These species generally grow weakly, forming only small colonies with strong greening haemolysis on selective media. They can grow at both 10 and 45 °C, in 6.5 % NaCl and are D group antigen negative (Pavel et al. 1984; Rahkila et al. 2011). Species in this group are typically adonitol-positive and L-sorbose-positive.

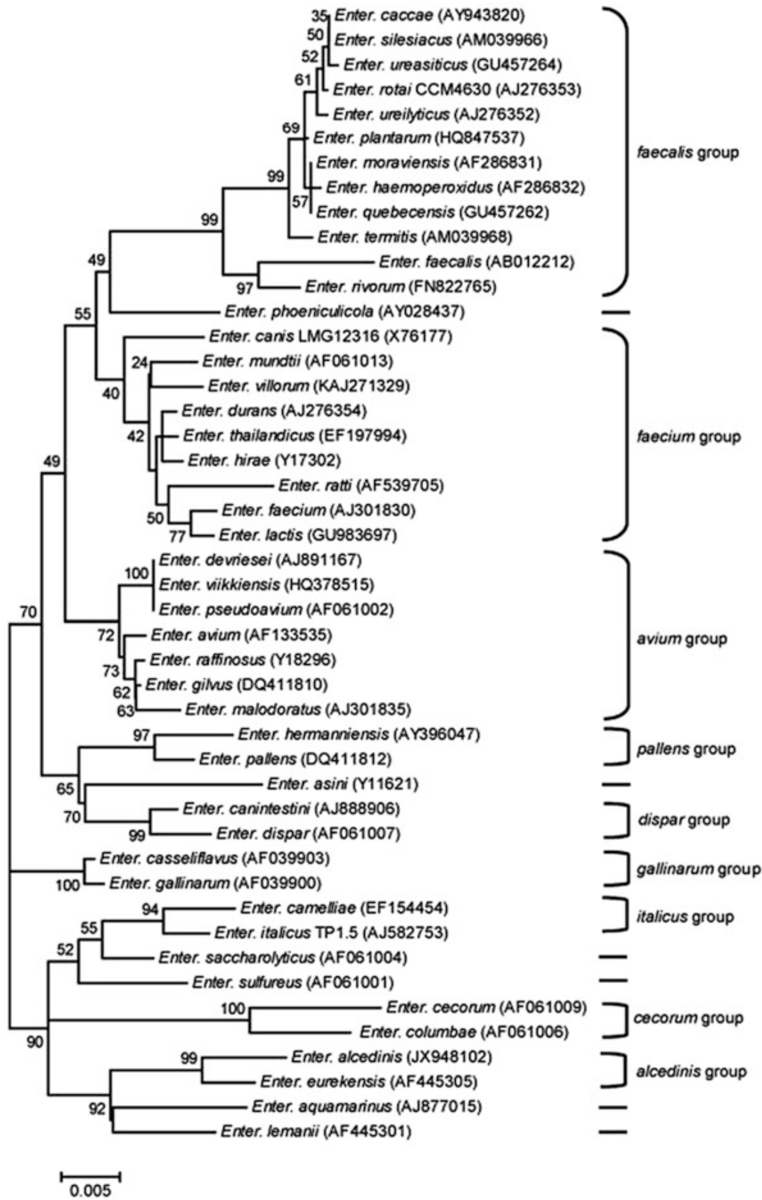


Fig. 1.2 Phylogenetic tree depicting the relationships between *Enterococcus* species based on 16S rRNA gene sequences. The tree was constructed through the neighbour-joining method with 1000 bootstrap replicates

1.3.4.4 Six Couples of *Enterococcus*

The *E. gallinarum* couple contains *E. casseliflavus* and *E. gallinarum*. Intrinsic low-level vancomycin resistance and motility are typical for *E. gallinarum*, although nonmotile strains have been rarely isolated on a few occassions (Clark et al. 1998; Patel et al. 1998). The *E. cecorum* couple comprises *E. cecorum* and *E. columbae*, which are more distantly phylogenetically related to each other than other pairs are, but have remarkable phenotypic similarities; they grow poorly on selective media but this can be greatly enhanced in a CO_2 atmosphere (Pavel et al. 1984). The *E. italicus* pair comprises *E. italicus* and *E. camelliae* which both have low biochemical activity compared with other species of *Enterococcus*. The remaining pairs are the *E. pallens* pair (*E. hermanniensis* and *E. pallens*), the *E. dispar* pair (*E. canintestini* and *E. dispar*) and the *E. alcedinis* pair (*E. alcedinis* and *E. eurekensis*).

1.3.5 Phylogeny and Evolution

To identify the species of *Enterococcus* accurately, alternative molecular methods have been successfully applied, such as (i) specific PCR amplification of rRNA intergenic spacers (Naimi et al. 1997), *ddl* and *van* genes (Satake et al. 1997; Kariyama et al. 2000), *ace* gene (Duh et al. 2001), *sod*A gene (Jackson et al. 2004), (ii) amplification of the putative transcriptional regulator gene Ef0027 (Liu et al. 2005), (iii) sequencing of the *ddl* genes (Ozawa et al. 2000), *cpn*60 gene (Goh et al. 2000) and *atp*A gene (Naser et al. 2005) and (iv) rep-PCR with the (GTG)5 primer (Svec et al. 2005). There have been numerous attempts to differentiate between human-derived isolates and food-derived isolates, mostly focusing on their DNA fingerprints. These studies have used a variety of molecular typing methods, such as amplified rDNA restriction analysis (ARDRA) (Ulrich and Müller 1998), pulsed-field gel electrophoresis (PFGE) (Descheemaeker et al. 1997; Vancanneyt et al. 2002), randomly amplified polymorphic DNA (RAPD) –PCR (Cocconcelli et al. 1995; Martin et al. 2005) and amplified fragment length polymorphisms (AFLP) (Vancanneyt et al. 2002; Willems et al. 2000). PFGE has been successfully used to show differences between clinical and food isolates (Klare et al. 1995), and between isolates from poultry and hospitalised human patients (van den Braak et al. 1998; Lemcke and Bulte 2000). Although PFGE is the gold standard method for distinguishing between isolates in the same species, it is considered expensive, time-consuming and complex (Ogier and Serror 2008). Recently, two methods, multilocus sequence typing (MLST), based on the nucleotide sequences of housekeeping genes, and multilocus variable analysis (MLVA), based on the variable-number tandem repeats, have proven more discriminatory than PFGE (Homan et al. 2002; Top et al. 2004; Titze-de-Almeida et al. 2004).

Homan et al. (2002) and Nallapareddy et al. (2002) first developed MLST scheme for the study of genetic diversity amongst isolates of *Enterococcus*. In the study of Homan et al., 139 isolates of *E. faecium* were divided into 62 STs based on 7 housekeeping genes (Homan et al. 2002). Subsequently, Bonora et al. (2004) also developed a MLST scheme based on seven housekeeping genes, *adk*, *atp*A, *ddl*, *gyd*, *gdh*, *pur*K and *pst*S. In their research, 9 STs were indentified for 14 vancomycin-resistant *E. faecium* isolates. In 2006, Camargo et al. (2006) determined the genetic relatedness of 56 vancomycin-resistant and vancomycin-sensitive *E. faecium* isolates identifying 26 STs within three groups with related allelic profiles. In 2006, Titze-de-Almeida et al. (2006) reported a genetic relationship between vancomycin-susceptible and vancomycin-resistant *E. faecium*. They found that a Brazilian outbreak clone did not cluster, as expected, in the previously named complex-17 by comparison with the allelic profiles of 139 *E. faecium* from different geographical regions and origins. The majority of Brazilian isolates, including the Brazilian outbreak clone, clustered apart from the *E. faecium* isolates from the database, suggesting that they had a distinct evolutionary history.

By sequencing 1 housekeeping gene (*pyr*C) and 3 *E. faecalis* antigen-encoding genes (*ace*, *efa*A and *sal*A) in 22 *E. faecalis* isolates 13 distinct STs were identified in the study of Nallapareddy et al. (2002). Ruiz-Garbajosa et al. (2006) also used a MLST scheme based on 7 housekeeping genes to investigate the epidemiology and population structure of 110 *E. faecalis* isolates from different sources and geographic locations. They revealed 55 different STs that grouped into four major CCs. Two CCs were particularly associated with the hospital environment. Furthermore, identical alleles were also found in genetically diverse isolates with no linkage disequilibrium, while the different MLST loci gave incongruent phylogenetic trees. This demonstrates that recombination was an important mechanism driving genetic variation in *E. faecalis* (Ruiz-Garbajosa et al. 2006).

MLVA is based on differences in the variable number of tandem repeats (VNTR) on multiple loci of the bacterial chromosome, that can be detected rapidly by PCR using specific primers based on the flanking regions of the tandem repeats (Nadon et al. 2013). Since MLVA types (MTs) are discriminated by gain and loss of discrete repeats, MLVA also provides an unambiguous assignment and nomenclature for genotypes, making it a portable technique suitable for data exchange. Top et al. (2004) described the development of MLVA and six VNTR loci, as a novel typing method to investigate genetic relatedness in 392 *E. faecium* isolates. These isolates had been recovered from different animals, the human community, hospital surveys and some were clinical isolates; they could be divided into 127 different MLVA profiles. The results showed that MLVA typing was as discriminatory as MLST and capable of recognising previously identified host-specific genogroups as accurately as MLST. In 2007, Werner et al. (2007) described phylogenetic relatedness of 51 *E. faecium* isolates based on MLST, MLVA and PFGE. They found that MLVA is an acceptable method to assign isolates of *E. faecium* to distinct CCs, but to investigate outbreaks, the available MLVA typing protocol was not discriminatory enough to be recommended as a standard that was superior to PFGE.

In a very large study, 411 *E. faecium* isolates from different continents and ecological niches were analysed by MLST, revealing that most hospital-derived vancomycin-resistant isolates belonged to a single clonal lineage called CC17 (Willems et al. 2005). MLST thus provides an excellent tool for epidemiological analysis of *E. faecium* isolates and, together with MLVA, may be a good alternative to PFGE. Despite the wide range of molecular typing methods available, and the emergence of MLST, it remains difficult to confidently discriminate between food-derived and clinical isolates.

In 2013, 40 isolates of *Enterococcus*, 8 *E. faecium* and 32 *E. faecalis*, were evaluated using MLST protocols and five housekeeping genes (*pep*C, *clp*X, *rec*A, *rpo*B and *gro*EL) by a research team in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P. R. China (Sun et al. unpublished data). The isolates were from naturally fermented yogurt in Inner Mongolia, Qinghai and Tibet of China. Twenty STs clustered into 4 CCs were identified. Totally 8 *E. faecium* contained 6 STs and 32 *E. faecalis* contained 14 STs. The most represented STs were ST5 found in seven isolates and ST6 found in six isolates. To explore the relationships amongst the 40 enterococcal isolates at the microevolutionary level, allelic profile-based phylogenetic analysis was performed using the minimum-spanning tree (Fig. 1.3). The minimum-spanning tree analysis showed that the evolution of these isolates was correlated with geographic environment, such as climate and altitude, and this is probably because isolates from the same region are likely to have been exposed to similar environmental selective pressures.

The split graphs of each locus showed treelike or slight network structures, except for *rpo*B and *rec*A, where some networks were detected, indicating a low rate of mutation and rare recombination in these housekeeping genes (Fig. 1.4a). This result was not consistent with previous reports and may be due to the isolates examined which were from a relatively homogeneous environment.

1.4 The Genus *Lactobacillus*

1.4.1 History

The genus *Lactobacillus* consists of Gram-positive, nonspore-forming rods that are usually nonmotile and occasionally nitrate reducers (Hammes and Vogel 1995; Hammes and Hertel 2006). They are catalase negative when growing in complex nutritional environments, such as carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins. *Lactobacillu*s species generally utilise glucose by fermentatively via the Embden-Meyerhof-Parnas pathway (EMP) or glycolysis, and may be either homofermentative, producing more than 85 % lactic acid from glucose, or heterofermentative, producing lactic acid, CO_2, ethanol and/or acetic acid in equimolar amounts.

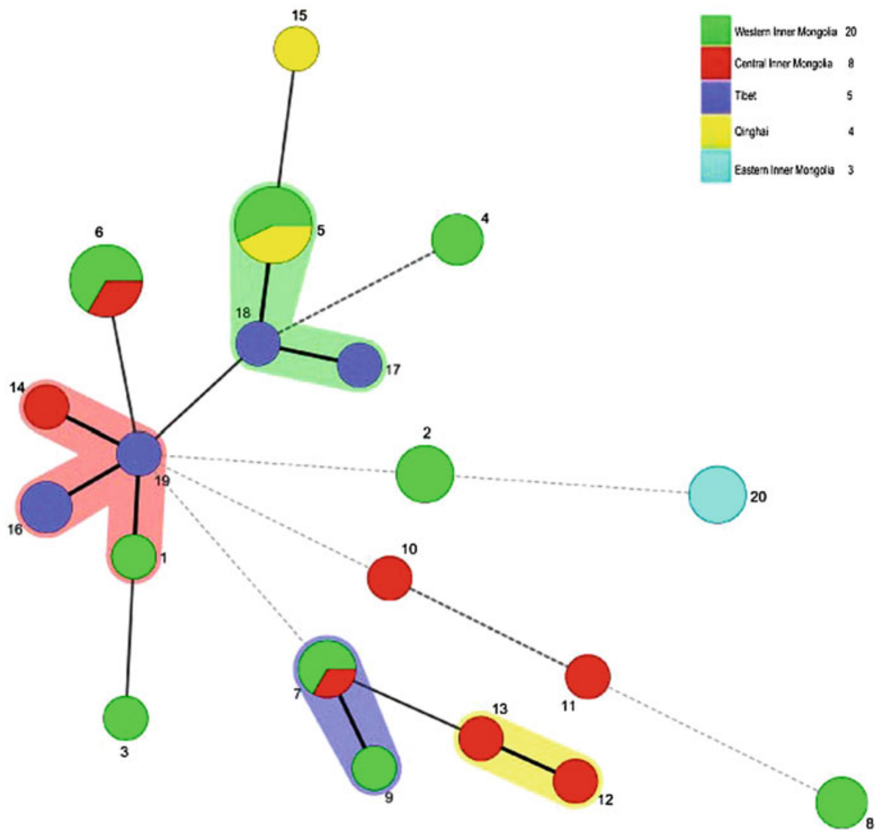


Fig. 1.3 Minimum spanning tree analysis of 40 enterococcal isolates based on allelic profiles at the five genes. Each circle corresponds to a ST, and the circle size denotes the number of strains sharing the same ST. Coloured zones between some groups of circles indicate that these profiles belong to the same clonal complex (Sun et al. unpublished data)

In 1896, the genus *Lactobacillus* was first described by Beijerinck (1901), and the type species was *Lactobacillus delbrueckii*. The genus *Lactobacillus* is the largest, most diverse group among the LAB, and currently comprised 158 validated species, seven of these species are comprised of 18 subspecies in total (Table 1.2, http://www.bacterio.net/l/lactobacillus.html, 20 December 2013). While *Lactobacillus* can be found in diverse habitats, they can be characterised within eight niche types according to the isolation source: plant (isolated from plant or plant-associated fermentation products), sourdough, meat products, dairy products, wine products, animal GI (isolated from human or animal

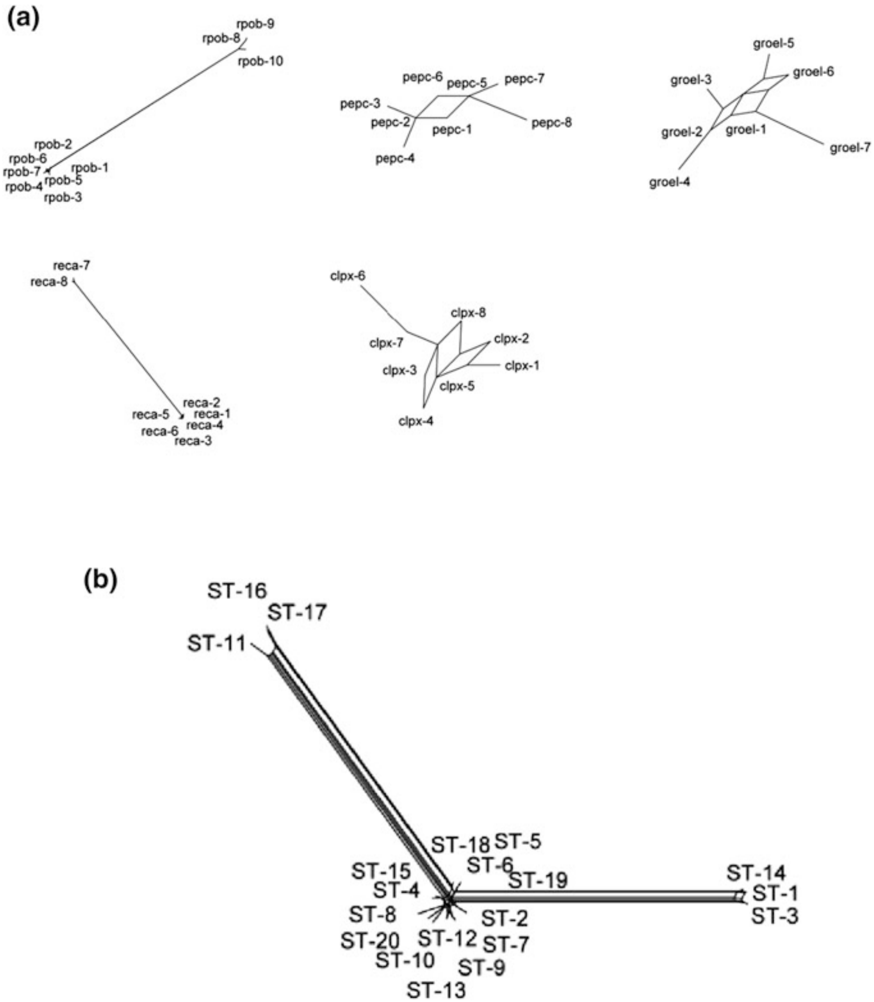


Fig. 1.4 Split decomposition analysis based on the allelic profiles of the *Enterococcus* strains. **a**: Split decomposition of alleles for individual MLST loci. **b**: Combined split decomposition of alleles for all ten MLST loci (Sun et al. unpublished data)

gastrointestinal tracts, including strains from saliva and faeces), animal-NGI (other human and animal-derived isolates with the exclusion of GI isolates) and the environment in general (Fig. 1.5).

DNA G+C content (mol.%): 32–55.

Type species: *Lactobacillus delbrueckii* (Leichmann 1896) Beijerinck (1901).

Table 1.2 List of the species in the genus *Lactobacillus*

Number	Species	Isolated time	Source classification	Metabolism*	Phylogroup**
1	*L. acetotolerans*	1986	Plant	FHE	delbrueckii
2	*L. acidifarinae*	2005	Sourdough	OHE	brevis
3	*L. acidipiscis*	2000	Meat products	FHE	salivarius
4	*L. acidophilus*	1970	Animals-NGI	OHO	delbrueckii
5	*L. agilis*	1982	Environment	FHE	salivarius
6	*L. algidus*	2000	Meat products	FHE	ss
7	*L. alimentarius*	1983	Meat products	FHE	alimentarius
8	*L. amylolyticus*	1999	Wine products	OHO	delbrueckii
9	*L. amylophilus*	1981	Plant	OHO	delbrueckii
10	*L. amylotrophicus*	2006	Plant	OHO	delbrueckii
11	*L. amylovorus*	1981	Plant	OHO	delbrueckii
12	*L. animalis*	1983	Animals-GI	OHO	salivarius
13	*L. antri*	2005	Animals-GI	OHE	reuteri
14	*L. apodemi*	2006	Animals-GI	FHE	salivarius
15	*L. aquaticus*	2009	Environment	OHO	salivarius
16	*L. aviarius* ssp. *araffinosus*	1986	Animals-GI	OHO	salivarius
17	*L. aviarius* ssp. *aviarius*	1985	Animals-GI	OHO	salivarius
18	*L. backii*	2013	Plant	OHO	backii-iwatensis
19	*L. bifermentans*	1943	Dairy products	FHE	coryniformis
20	*L. brantae*	2012	Animals-GI	FHE	brantae-saniviri
21	*L. brevis*	1934	Animals-GI	OHE	brevis
22	*L. buchneri*	1923	Plant	OHE	buchneri
23	*L. cacaonum*	2009	Plant	FHE	salivarius
24	*L. camelliae*	2007	Plant	OHO	ss
25	*L. capillatus*	2008	Plant	OHO	salivarius
26	*L. casei*	1971	Dairy products	FHE	casei
27	*L. ceti*	2008	Animals-NGI	FHE	salivarius
28	*L. coleohominis*	2001	Animals-NGI	FHE	reuteri
29	*L. collinoides*	1972	Plant	OHE	collinoides
30	*L. composti*	2007	Plant	FHE	ss
31	*L. concavus*	2005	Environment	OHO	concavus-dextrinicus
32	*L. coryniformis* ssp. *coryniformis*	1965	Plant	FHE	coryniformis
33	*L. coryniformis* ssp. *torquens*	1965	Environment	FHE	coryniformis
34	*L. crispatus*	1970	Animals-NGI	OHO	delbrueckii
35	*L. crustorum*	2007	Sourdough	OHO	alimentarius
36	*L. curieae*	2013	Plant	OHO	curieae-senioris
37	*L. curvatus*	1996	Dairy products	FHE	sakei
38	*L. delbrueckii* ssp. *bulgaricus*	1984	Dairy products	OHO	delbrueckii

(continued)

Table 1.2 (continued)

Number	Species	Isolated time	Source classification	Metabolism*	Phylogroup**
39	*L. delbrueckii* ssp. *delbrueckii*	1901	Plant	OHO	delbrueckii
40	*L. delbrueckii* ssp. *indicus*	2005	Dairy products	OHO	delbrueckii
41	*L. delbrueckii* ssp. *jakobsenii*	2013	Plant	OHO	delbrueckii
42	*L. delbrueckii* ssp. *lactis*	1984	Dairy products	OHO	delbrueckii
43	*L. delbrueckii* ssp. *sunkii*	2012	Plant	OHO	delbrueckii
44	*L. dextrinicus*	2009	Plant	OHE	concavus-dextrinicus
45	*L. diolivorans*	2002	Plant	OHE	buchneri
46	*L. equi*	2002	Animals-GI	OHO	salivarius
47	*L. equicursoris*	2010	Animals-NGI	OHO	delbrueckii
48	*L. equigenerosi*	2008	Animals-NGI	OHE	reuteri
49	*L. fabifermentans*	2009	Plant	FHE	plantarum
50	*L. farciminis*	1983	Meat products	OHO	alimentarius
51	*L. farraginis*	2007	Wine products	FHE	buchneri
52	*L. fermentum*	1901	Plant	OHE	reuteri
53	*L. floricola*	2011	Plant	OHO	ss
54	*L. florum*	2010	Plant	OHE	fructivorans
55	*L. fornicalis*	2000	Animals-NGI	FHE	delbrueckii
56	*L. fructivorans*	1934	Plant	OHE	fructivorans
57	*L. frumenti*	2000	Sourdough	OHE	reuteri
58	*L. fuchuensis*	2002	Meat products	FHE	sakei
59	*L. futsaii*	2012	Plant	OHO	alimentarius
60	*L. gallinarum*	1992	Animals-GI	OHO	delbrueckii
61	*L. gasseri*	1980	Animals-NGI	OHO	delbrueckii
62	*L. gastricus*	2008	Animals-GI	OHE	reuteri
63	*L. ghanensis*	2007	Plant	OHO	salivarius
64	*L. gigeriorum*	2012	Animals-GI	OHO	delbrueckii
65	*L. graminis*	1989	Plant	FHE	sakei
66	*L. hammesii*	2005	Sourdough	FHE	brevis
67	*L. hamsteri*	1988	Animals-GI	FHE	delbrueckii
68	*L. harbinensis*	2006	Plant	FHE	perolens
69	*L. hayakitensis*	2007	Animals-GI	OHO	salivarius
70	*L. heilongjiangensis*	2013	Plant	ND	alimentarius
71	*L. helveticus*	1925	Dairy products	OHO	delbrueckii
72	*L. hilgardii*	1936	Wine products	OHE	buchneri
73	*L. hominis*	2013	Animals-GI	OHO	delbrueckii
74	*L. homohiochii*	1957	Meat products	FHE	fructivorans
75	*L. hordei*	2008	Plant	OHO	salivarius
76	*L. iners*	1999	Animals-NGI	OHO	delbrueckii
77	*L. ingluviei*	2003	Animals-GI	OHE	reuteri
78	*L. intestinalis*	1990	Animals-GI	FHE	delbrueckii

(continued)

Table 1.2 (continued)

Number	Species	Isolated time	Source classification	Metabolism*	Phylogroup**
79	*L. iwatensis*	2013	Plant	OHO	backii-iwatensis
80	*L. jensenii*	1970	Animals-NGI	FHE	delbrueckii
81	*L. johnsonii*	1992	Animals-NGI	OHO	delbrueckii
82	*L. kalixensis*	2005	Animals-GI	OHO	delbrueckii
83	*L. kefiranofaciens* ssp. *kefiranofaciens*	1988	Plant	OHO	delbrueckii
84	*L. kefiranofaciens* ssp. *kefirgranum*	2004	Plant	OHO	delbrueckii
85	*L. kefiri*	1983	Plant	OHE	buchneri
86	*L. kimchicus*	2008	Plant	FHE/OHE	collinoides
87	*L. kimchiensis*	2013	Plant	OHO	alimentarius
88	*L. kisonensis*	2009	Plant	OHE	buchneri
89	*L. kitasatonis*	2003	Animals-GI	OHO	delbrueckii
90	*L. koreensis*	2011	Plant	OHE	brevis
91	*L. kunkeei*	2012	Wine products	OHE	kunkeei-ozensis
92	*L. lindneri*	1997	Plant	OHE	fructivorans
93	*L. malefermentans*	1989	Wine products	OHE	ss
94	*L. mali*	1970	Plant	OHO	salivarius
95	*L. manihotivorans*	1998	Plant	OHO	manihotivorans
96	*L. mindensis*	2003	Sourdough	OHO	alimentarius
97	*L. mucosae*	2000	Animals-GI	OHE	reuteri
98	*L. murinus*	1982	Animals-GI	FHE	salivarius
99	*L. nagelii*	2000	Wine products	OHO	salivarius
100	*L. namurensis*	2007	Sourdough	OHE	brevis
101	*L. nantensis*	2006	Sourdough	FHE	alimentarius
102	*L. nasuensis*	2012	Plant	OHO	manihotivorans
103	*L. nodensis*	2009	Plant	FHE	alimentarius
104	*L. odoratitofui*	2010	Plant	OHE	collinoides
105	*L. oeni*	2009	Wine products	OHO	salivarius
106	*L. oligofermentans*	2005	Meat products	OHE	vaccinostercus
107	*L. oris*	1988	Animals-GI	OHE	reuteri
108	*L. oryzae*	2013	Plant	OHE	manihotivorans
109	*L. otakiensis*	2009	Plant	OHE	buchneri
110	*L. ozensis*	2011	Plant	OHE	kunkeei-ozensis
111	*L. panis*	1996	Sourdough	OHE	reuteri
112	*L. pantheris*	2002	Animals-GI	OHO	pantheris-thailandensis
113	*L. parabrevis*	2006	Plant	OHE	brevis
114	*L. parabuchneri*	2002	Animals-GI	OHE	buchneri
115	*L. paracasei* ssp. *paracasei*	1989	Dairy products	FHE	casei
116	*L. paracasei* ssp. *tolerans*	1989	Dairy products	FHE	casei

(continued)

Table 1.2 (continued)

Number	Species	Isolated time	Source classification	Metabolism*	Phylogroup**
117	*L. paracollinoides*	2004	Environment	OHE	collinoides
118	*L. parafarraginis*	2007	Wine products	FHE	buchneri
119	*L. parakefiri*	1994	Dairy products	OHE	buchneri
120	*L. paralimentarius*	1999	Sourdough	FHE	alimentarius
121	*L. paraplantarum*	1996	Wine products	FHE	plantarum
122	*L. pasteurii*	2013	Animals-GI	FHE	delbrueckii
123	*L. paucivorans*	2010	Environment	OHE	brevis
124	*L. pentosus*	1987	Plant	FHE	plantarum
125	*L. perolens*	2000	Plant	FHE	perolens
126	*L. plantarum* ssp. *argentoratensis*	2005	Plant	FHE	plantarum
127	*L. plantarum* ssp. *plantarum*	1923	Plant	FHE	plantarum
128	*L. pobuzihii*	2010	Plant	FHE	salivarius
129	*L. pontis*	1994	Sourdough	OHE	reuteri
130	*L. porcinae*	2013	Environment	OHO	manihotivorans
131	*L. psittaci*	2001	Animals-NGI	OHE	delbrueckii
132	*L. rapi*	2009	Plant	OHE	buchneri
133	*L. rennini*	2006	Animals-NGI	FHE	coryniformis
134	*L. reuteri*	1982	Animals-GI	OHE	reuteri
135	*L. rhamnosus*	1989	Dairy products	FHE	casei
136	*L. rossiae*	2005	Sourdough	OHE	rossiae-siliginis
137	*L. ruminis*	1973	Animals-NGI	OHO	salivarius
138	*L. saerimneri*	2004	Animals-GI	OHO	salivarius
139	*L. sakei* ssp. *carnosus*	1996	Meat products	FHE	sakei
140	*L. sakei* ssp. *sakei*	1996	Wine products	FHE	sakei
141	*L. salivarius*	2006	Animals-GI	FHE	salivarius
142	*L. sanfranciscensis*	1984	Sourdough	OHE	fructivorans
143	*L. saniviri*	2012	Animals-GI	FHE	brantae-saniviri
144	*L. satsumensis*	2005	Wine products	OHO	salivarius
145	*L. secaliphilus*	2007	Sourdough	FHE	reuteri
146	*L. selangorensis*	2011	Plant	OHO	ss
147	*L. senioris*	2012	Animals-GI	OHE	curieae-senioris
148	*L. senmaizukei*	2008	Plant	FHE	brevis
149	*L. sharpeae*	1982	Environment	OHO	ss
150	*L. shenzhenensis*	2013	Dairy products	OHE	perolens
151	*L. siliginis*	2006	Sourdough	OHE	rossiae-siliginis
152	*L. similis*	2010	Plant	OHE	collinoides
153	*L. spicheri*	2004	Sourdough	FHE	brevis
154	*L. sucicola*	2009	Plant	OHO	salivarius
155	*L. suebicus*	1989	Plant	OHE	vaccinostercus

(continued)

Table 1.2 (continued)

Number	Species	Isolated time	Source classification	Metabolism*	Phylogroup**
156	*L. sunkii*	2009	Plant	OHE	buchneri
157	*L. taiwanensis*	2009	Plant	OHO	delbrueckii
158	*L. thailandensis* thailandensis	2007	Plant	OHO	pantheris-
159	*L. tucceti*	2009	Meat products	OHO	alimentarius
160	*L. ultunensis*	2005	Animals-GI	OHO	delbrueckii
161	*L. uvarum*	2009	Plant	OHO	salivarius
162	*L. vaccinostercus*	2006	Animals-NGI	OHE	vaccinostercus
163	*L. vaginalis*	1989	Animals-NGI	OHE	reuteri
164	*L. versmoldensis*	2003	Meat products	OHO	alimentarius
165	*L. vini*	2006	Plant	FHE	salivarius
166	*L. xiangfangensis*	2012	Plant	FHE	plantarum
167	*L. yonginensis*	2013	Plant	ND	brevis
168	*L. zeae*	1996	Wine products	FHE	casei
169	*L. zymae*	2005	Sourdough	OHE	brevis

The table is compiled from the studies of Salvetti et al. (2012)
* OHO, obligately homofermentative; FHE, facultatively heterofermentative; OHE, obligately heterofermentative; ND, no data available
** ss, single species

1.4.2 Cell Morphology and Cultural Characteristics

Lactobacillus species are generally anaerobic, but can be aerotolerant, and aciduric or acidophilic. In general, they do not synthesize porphyrinoids and are devoid of heme-dependent activities (Hammes and Vogel 1995). Cells vary from long and slender, sometimes bent rods, to short, often coryneform, cocci; chain formation is common in rod-shaped forms and these chains are usually regular in width and length (0.5−1.2 × 1.0−10.0 μm). For growth, the temperature range is between 2 and 53 °C, and the pH range is between 3 and 8. Optimal growth temperature and pH are usually 30–40 °C and 5.5–6.2, respectively (Salvetti et al. 2012; Hammes and Hertel 2009).

1.4.3 Phylogenetic Position of the Lactobacillus Genus

The genus *Lactobacillus*, together with the genera *Paralactobacillus* and *Pediococcus*, belong to the family *Lactobacillaceae* and order *Lactobacillales* in the Firmicutes (Hammes and Hertel 2009). Although species in the genera *Pediococcus, Leuconostoc* and *Lactobacillus* are closely related, they will be described

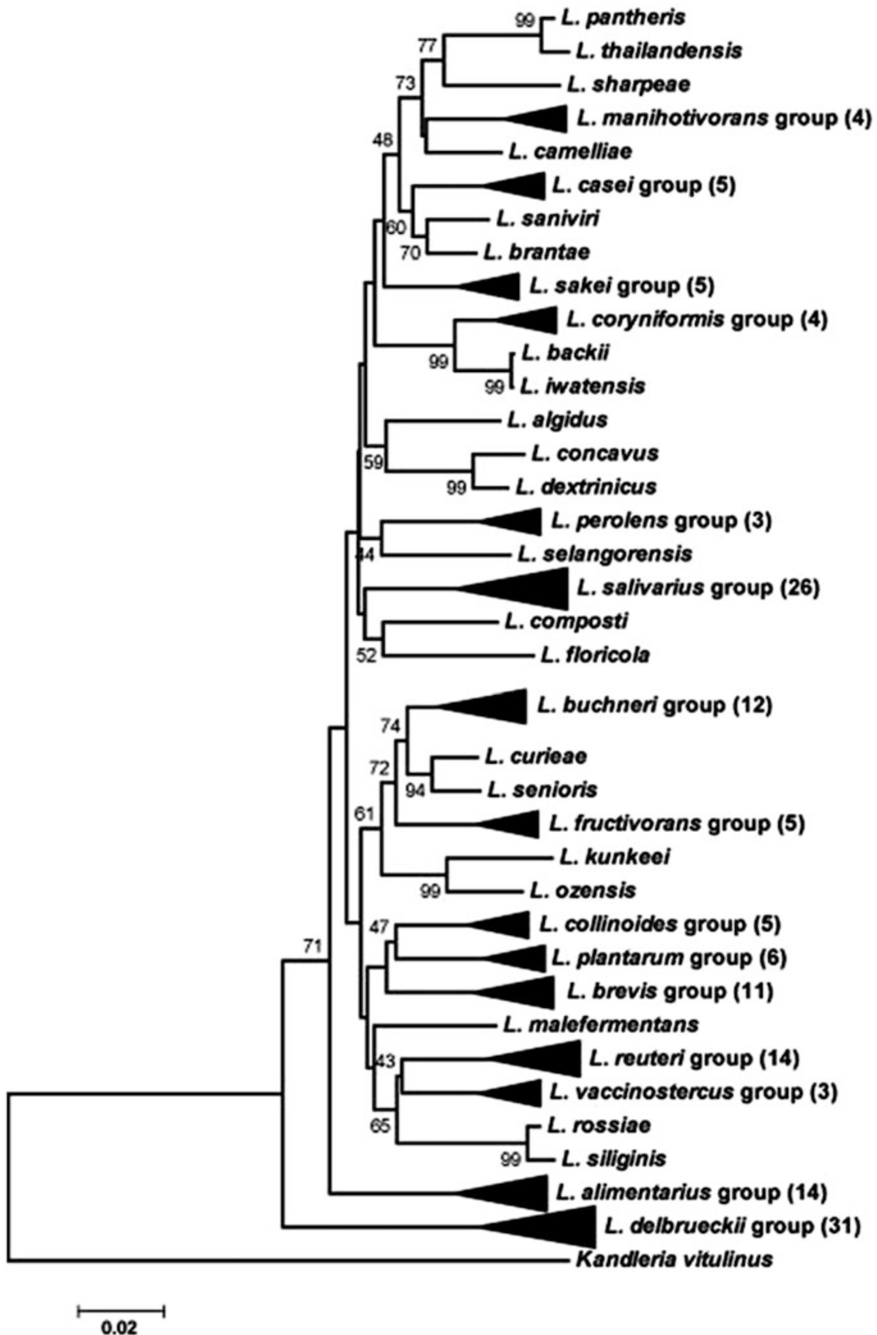


Fig. 1.5 Phylogenetic tree depicting the relationships between *Lactobacillus* species based on 16S rRNA gene sequence. The tree was constructed using the neighbour-joining method with 1000 bootstrap replicates

separately elsewhere. Using 16S rRNA gene sequence analysis, the phylogeny of LAB have been studied extensively in the past (Hammes and Hertel 2006) revealing some of the relationships between *Lactobacillus*, *Leuconostoc* and *Pediococcus* (Schleifer and Ludwig 1995a; Stiles and Holzapfel 1997).

1.4.4 Grouping Within the Genus Lactobacillus

Originally, species in the genus *Lactobacillus* were grouped based on their growth temperature and ability to ferment hexoses, and subsequently according to their homo/heterofermentative potential (Carr et al. 2002). These subdivisions were revisited by Pot and colleagues (Pot et al. 1994), but the accepted 'modern' definition is the one given by Hammes and Vogel (Hammes and Vogel 1995) which divides the genus into obligate homofermentative, facultative heterofermentative and obligate heterofermentative species, based on the types of sugars fermented and the fermentation process used. The obligate homofermentative (OHO) species only ferment hexoses and almost exclusively (85 %) to lactic acid via the EMP pathway or glycolysis; pentoses and gluconate are not fermented. Facultative heterofermentative (FHE) species ferment hexoses to lactic acid via EMP but, under glucose limitation, are also able to degrade pentoses and gluconate via an inducible phosphoketolase, an enzyme of the pentose phosphate (PP) pathway, resulting in production of acetic acid, ethanol and formic acid. Finally, the obligate heterofermentative (OHE) species possess a FDB aldolase, but not phosphoketolase, and they metabolise pentoses and hexoses exclusively via the phosphogluconate pathway (corre-sponding to the first part of the PP) and produce lactic acid, ethanol (or acetic acid) and CO_2 (Hammes and Vogel 1995).

There are 169 recognised species in the genus *Lactobacillus* divided amongst three metabolism groups: OHO, FHE and OHE. Within these three groups, the species are further arranged according to their phylogenetic relationships; based on their 16S rRNA gene sequences they are divided into 15 large phylogenetic groups, seven pairs (small phylogenetic groups containing only two species) and seven singletons each represented by a single species (Table 1.2, Fig. 1.5).

1.4.4.1 *Lactobacillus delbrueckii* Group

The *Lactobacillus delbrueckii* group comprises of 33 species and subspecies; *L. acetotolerans*, *L. acidophilus*, *L. amylolyticus*, *L. amylotrophicus*, *L. amylophilus*, *L. amylovorus*, *L. crispatus*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *indicus*, *L. delbrueckii* subsp. *jakobsenii*, *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *sunkii*, *L. equicursoris*, *L. fornicalis*, *L. gallinarum*, *L. gasseri*, *L. gigeriorum*, *L. hamster*, *L. helveticus*, *L. hominis*, L. *iners*, *L. intestinalis*, *L. jensenii*, *L. johnsonii*, *L. kalixensis*, *L. kefiranofaciens* subsp. *kefiranofaciens*, *L. kefiranofaciens* subsp. *kefirgranum*, *L.*

kitasatonis, *L. pasteurii*, *L. psittaci*, *L. taiwanensis* and *L. ultunensis* (Table 1.2, Fig. 1.5).

Taxonomically this is the most important phylogenetic group due to the presence of *L. delbrueckii*, the type species of the genus, which the name *Lactobacillus* is permanently linked to. It contains mainly the OHOs (27 out of 33), but also some FHEs species (6 out of 33, Table 1.2). The GC content ranges between 33 and 51 mol.%, which may be explained by changes in codon usages due to degeneration of the genetic code (Hammes and Hertel 2009; Schleifer and Ludwig 1995b). The majority of the species are characterised as Lys-D-Asp peptidoglycan types. This group is very heterogeneous in terms of isolation source (Table 1.2).

1.4.4.2 *Lactobacillus salivarius* Group

The *Lactobacillus salivarius* group comprises 26 species and subspecies; *L. acidipiscis*, *L. agilis*, *L. animalis*, *L. apodemi*, *L. aquaticus*, *L. aviarius* subsp. *araffinosus*, *L. aviarius* subsp. *aviaries*, *L. cacaonum*, *L. capillatus*, *L. ceti*, *L. equi*, *L. ghanensis*, *L. hayakitensis*, L. *hordei*, *L. mali*, *L. murinus*, *L. nagelii*, *L. oeni*, *L. pobuzihii*, *L. ruminis*, *L. saerimneri*, *L. salivarius*, *L. satsumensis*, *L. sucicola*, *L. uvarum* and *L. vini* (Fig. 1.5). As with the *L. delbrueckii* group, this group contains mainly OHOs (17 out of 26 species) but also some FHEs species (9 out of 26, Table 1.2). The GC content ranges between 32 and 44 mol%. Each species produces L(+)-lactic acid or both L(+)- and D(−)-lactic acids, but no one produces the D(−)-isomer exclusively. They are all either Lys-D-Asp or meso-Dpm-direct (Hammes and Hertel 2009). Interestingly, this group includes the majority of the motile *Lactobacillus* species,such as *L. agilis*, *L. aquaticus*, *L. capillatus*, *L. ghanensis*, *L. mali*, *L. nagelii*, *L. oeni*, *L. ruminis*, *L. sucicola*, *L. satsumensis*, *L. uvarum* and *L. vini* (Salvetti et al. 2012).

1.4.4.3 *Lactobacillus reuteri* Group

The *Lactobacillus reuteri* group comprises 14 species; *L. antri*, *L. coleohominis*, *L. equigenerosi*, *L. fermentum*, *L. frumenti*, *L. gastricus*, *L. ingluviei*, *L. mucosae*, *L. oris*, *L. panis*, *L. pontis*, *L. reuteri*, *L. secaliphilus* and *L. vaginalis* (Table 1.2). Previously *L. rossiae*, *L. suebicus* and *L. vaccinostercus* have been defined as *L. reuteri* group (Hammes and Hertel 2006) but they have subsequently been transferred to other groups (Salvetti et al. 2012). This group is mainly characterised by OHO species, with the exception of *L. coleohominis* and *L. secaliphilus*, which are FHE species. Species in this group exhibit a wide range of GC contents (38–56 mol.%). The peptidoglycan types are Lys-D-Asp, Orn-D-Asp or meso-Dpm-direct (Hammes and Hertel 2009).

1.4.4.4 *Lactobacillus buchneri* Group

The *Lactobacillus buchneri* group comprises by 12 species, *L. buchneri*, *L. diolivorans*, *L. farraginis*, *L. hilgardii*, *L. kefiri*, *L. kisonensis*, *L. otakiensis*, *L. parabuchneri*, *L. parafarraginis*, *L. parakefiri*, *L. rapi* and *L. sunkii*. As with the *L. reuteri* group, they are mainly OHO species, with the exception of *L. farraginis* and *L. parafarraginis* which are FHE species. The GC content is typically between 38.8 and 42 mol%, and they are mostly Lys–D-Asp peptidoglycan types. All species produce both L(+)- and D(−)- lactic acids, with the exception of *Lb. parakefiri*, which produces only L(+)-lactic acid (Salvetti et al. 2012; Hammes and Hertel 2009).

1.4.4.5 *Lactobacillus alimentarius* Group

The *Lactobacillus alimentarius* group comprises by 12 species: *L. alimentarius*, *L. crustorum*, *L. farciminis*, *L. futsaii*, *L. heilongjiangensis*, *L. kimchiensis*, *L. mindensis*, *L. nantensis*, *L. nodensis*, *L. paralimentarius*, *L. tucceti* and *L. versmoldensis*. Species in this group are either OHO or FHE, and the GC content ranges between 35 and 40 mol%. They are mainly characterised as Lys-D-Asp peptidoglycan type, with the exception of *L. tucceti* which is a Lys-Gly-D-Asp peptidoglycan type (Hammes and Hertel 2009; Chenoll et al. 2006). Interestingly, the majority of the species were isolated from traditional and commercially produced sourdough (Table 1.2).

1.4.4.6 *Lactobacillus brevis* Group

The *Lactobacillus brevis* group is constituted of 11 species: *L. acidifarinae*, *L. brevis*, *L. hammesii*, *L. koreensis*, *L. namurensis*, *L. parabrevis*, *L. paucivorans*, *L. senmaizukei*, *L. spicheri*, *L. yonginensis* and *L. zymae*. It contains both OHO and FHE species, and their GC content ranges between 46 and 55 mol%. They are all Lys-D-Asp peptidoglycan type. *L. koreensis* is the only motile species in the group (Bui et al. 2011). All species in this group are able to grow in NaCl concentrations between 5 and 10 % (Salvetti et al. 2012; Hammes and Hertel 2009).

1.4.4.7 *Lactobacillus collinoides* Group

The *Lactobacillus collinoides* group comprises five species: *L. collinoides*, *L. kimchicus*, *L. odoratitofui*, *L. paracollinoides* and *L. similis*. All species are OHE and their GC content is between 39.7 and 48.5 mol%. All species in this group are able to form D(−)-lactic acid and three species also produce the L(+) isomer (Hammes and Hertel 2009).

1.4.4.8 *Lactobacillus fructivorans* Group

The *Lactobacillus fructivorans* group comprises five species: *L. florum*, *L. fructivorans*, *L. homohiochii*, *L. lindneri* and *L. sanfranciscensis*. The majority of species are OHE, excect for *L. homohiochii* which is FHE. The GC content of species in this group is between 35 and 42 mol% and they are either Lys-D-Asp or Lys-Ala peptidoglycan types (Salvetti et al. 2012; Hammes and Hertel 2009).

1.4.4.9 *Lactobacillus plantarum* Group

The *Lactobacillus plantarum* group comprises six species and subspecies: *L. fabifermentans*, *L. paraplantarum*, *L. pentosus*, *L. plantarum* subsp. *argentoratensis*, *L. plantarum* subsp. *plantarum* and *L. xiangfangensis*. This group is very homogeneous in terms of metabolic features since all species are FHE. Their GC content ranges between 44 and 47 mol% and they are all meso-Dpm-direct peptidoglycan type (Hammes and Hertel 2009).

1.4.4.10 *Lactobacillus sakei* Group

The *Lactobacillus sakei* group comprises five species and subspecies: *L. curvatus*, *L. fuchuensis*, *L. graminis*, *L. sakei* subsp. *carnosus* and *L. sakei* subsp. *sakei*. All species are FHE and their GC content is between 41 and 44 mol%. They produce both D(−)- and L(+)-lactic acids with the exception of *L. fuchuensis*, which produces only L(+)-lactic acid (Hammes and Hertel 2009; Sakala et al. 2002b). This group is heterogeneous in terms of production of ammonia from arginine and acetoin (Salvetti et al. 2012).

1.4.4.11 *Lactobacillus casei* Group

The *Lactobacillus casei* group conprises five species and subspecies: *L. casei*, *L. paracasei* subsp. *paracasei*, *L. paracasei* subsp. *tolerans*, *L. rhamnosus*, *L. zeae*. This group is homogenous since all species are FHE, their GC content ranges between 45 and 47 mol% and they are all Lys- D-Asp peptidoglycan type. Furthermore, they are able to produce acetoin and all of them form L(+)-lactic acid (Salvetti et al. 2012; Hammes and Hertel 2009).

1.4.4.12 *Lactobacillus coryniformis* Group

The *Lactobacillus coryniformis* group consists of four species and subspecies: *L. bifermentans*, *L. coryniformis* subsp. *coryniformis*, *L. coryniformis* subsp. *torquens* and *L. rennin*. They are a homogeneous group since all are FHE, have a GC

content of 45 mol%, Lys-D-Asp peptidoglycan type and produce both D(−)- and L(+)-lactic acid isomers. None of them produce ammonia from arginine (Salvetti et al. 2012; Hammes and Hertel 2009).

1.4.4.13 *Lactobacillus manihotivorans* Group

The *Lactobacillus manihotivorans* group comprises four species: *L. porcinae*, *L. oryzae*, *L. manihotivorans* and *L. nasuensis*. They are all OHO and characterised by a GC content between 47.6 and 59.2 mol%. Differently from *L. manihotivorans* and *L. nasuensis*, *L. porcinae* produces only L(+)-lactic acid (Salvetti et al. 2012; Hammes and Hertel 2009). Interestingly, *L. nasuensis* is the only species able to grow at pH 8 (Cai et al. 2012).

1.4.4.14 *Lactobacillus perolens* Group

The *Lactobacillus perolens* group comprises three species *L. harbinensis*, *L. perolens* and *L. shenzhenensis*. *L. harbinensis* and *L. perolens* are FHE and produce only L(+)-lactic acid, while *L. shenzhenensis* is OHE (Zou et al. 2013). The GC content ranges from 45 up to 56 mol%.

1.4.4.15 *Lactobacillus vaccinostercus* Group

The *Lactobacillus vaccinostercus* group comprises three species: *L. oligofermentans*, *L. suebicus* and *L. vaccinostercus*. All species are OHE, their GC content is between 35.3 and 41 mol% and they are meso-Dpm-direct peptidoglycan type (Salvetti et al. 2012; Hammes and Hertel 2009).

1.4.4.16 Species Outside Groups

As for species outside the main groups, they comprise seven pairs and seven single lines of descent. The pairs are *L. backii*- *L. iwatensis*, *L. brantae*- *L. saniviri*, *L. concavus*- *L. dextrinicus*, *L. curieae*- *L. senioris*, *L. kunkeei*- *L. ozensis*, *L. pantheris*- *L. thailandensis* and *L. rossiae*-*L. siliginis* (Table 1.2 and Fig. 1.5).

1.4.5 Phylogeny of the Genus Lactobacillus *Based on Whole Genome Analysis*

Using *Lactococcus garvieae* and *Streptococcus mutans* as outgroups to define the position of the most recent common ancestor (MRCA), the maximum likelihood tree for the *Lactobacillus* complex was rebuilt based on 72 core genes (Fig. 1.6). In

agreement with previous observations based on 28 LAB genomes (Zhang et al. 2011), the *Lactobacillus* complex could be divided into two main branches after divergence from the MRCA. Such delineations are supported by the phylogenetic relationships constructed based on the amino acid sequences of core genes. Therefore, the results confirm the previous definitions of the phylogroups, with two exceptions which have led to revisions (Fig. 1.6). The first revision was to assign the single species *L. camelliae* to the *L. manihotivorans* group because it was closely phylogenetic related and resulted in the *Lb. manihotivorans* group being a monophyletic taxonomic unit. The second revision was the removal of *L. amylotrophicus* and *L. amylophilus*, from the *L. delbrueckii* group because the other species in the group were more closely related to each other (mean TNI value is 34.7 %) compared with the other two species (TNI value <19 %, Sun et al. unpublished data).

The genomic and evolutionary features of this specific branch in the tree of bacteria were preliminarily elucidated by whole genome sequencing and phylogenomic analysis of the type isolates for most of the *Lactobacillus* species. As different isolates of the same *Lactobacillus* species can exhibit different fermentation types and occupy multiple niches (Douillard et al. 2013), in this study, some observations may have been influenced by sampling bias because only the type isolate for each species was examined. Nevertheless, the evidence demonstrates that the OHO and OHE species are descended from FHE species and that the species isolated from animals exhibited different evolutionary patterns on the two main branches of the *Lactobacillus* tree. These results also indicate that similar genomic features may appear in species from different niches and that different genetic mechanisms may be adapted to fit the same niche.

1.4.6 Phylogeny and Evolution Based on Multilocus Sequence Typing

The usefulness of housekeeping genes in bacterial taxonomy and phylogeny has been reported for several species in the genus *Lactobacillus* (Blaiotta et al. 2008; Torriani et al. 2001; Felis et al. 2001; Chavagnat et al. 2002; Ventura et al. 2003a; Ilse et al. 2009; Kudo et al. 2012; Chao et al. 2012; Watanabe et al. 2009). For the *L. casei* group, *L. delbrueckii* group and *L. planrarum* group, partial sequences of the *hsp*60 (*gro*EL, encoding a 60-kDa heat shock protein), *phe*S (phenylalanyl-tRNA synthase alpha subunit), *rec*A (recombinase A), *rpo*A (α subunit of RNA polymerase), *rpo*B (β subunit of RNA polymerase) and *tuf* (encoding elongation factor Tu) genes used in combination with 16S rRNA gene sequences have been effective in distinguishing between isolates from the same species or similar phylogenetic background. Furthermore, application of these gene sequences has also been effective for identifying new species or subspecies, such as, *L. delbrueckii* subsp. *sunkii*, *L. futsaii*, *L. kisonensis*, *L. otakiensis*, *L. sunkii*. In the

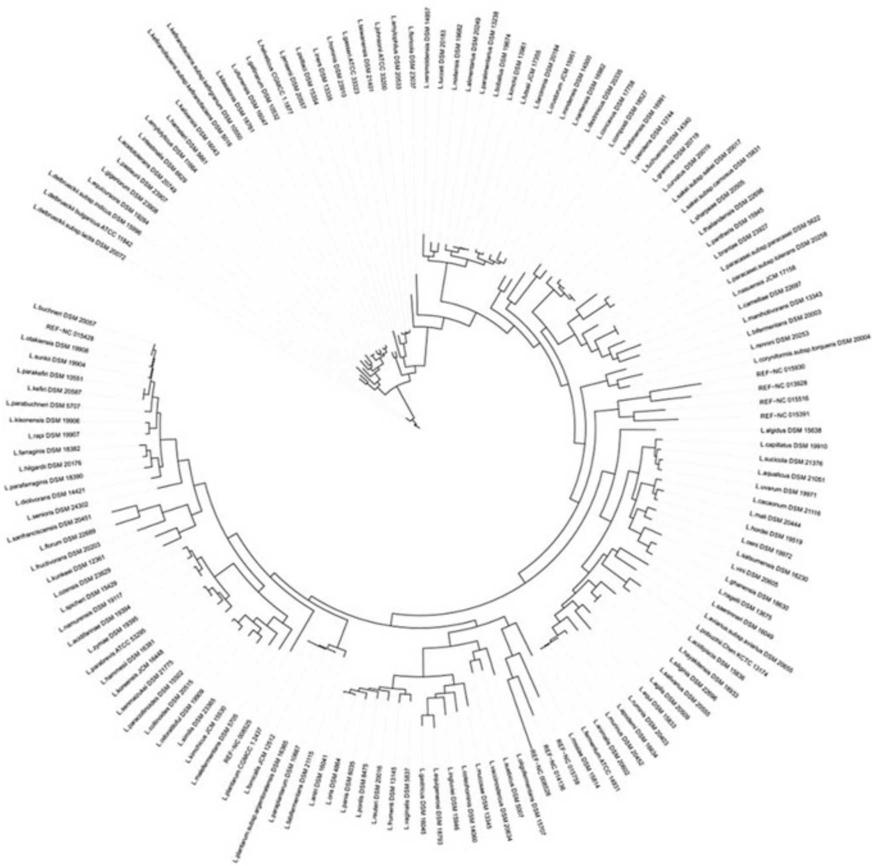


Fig. 1.6 Maximum likelihood tree of the *Lactobacillus* complex, based on the amino acid sequences of 72 core genes

Watanabe et al. study, four type strains of species from the genus *Lactobacillus* were described based on partial sequences of the *rec*A genes (Watanabe et al. 2009).

Recently, some microbiologists are recommending DNA sequence analysis of several housekeeping genes or other protein coding gene sequences in an approach called multilocus sequence typing (MLST) to discriminate between closely related species and analyse the genetic diversity and phylogenetic relationships between them. It was shown to be a powerful technique for bacterial typing (Maiden et al. 1998; Enright and Spratt 1999), providing critical information on evolutionary history, population structure and long-term epidemiology of bacterial species (Maiden et al. 1998). Although, principally, MLST has been used to study major bacterial pathogens, several recent MLST schemes have also been developed for species in the genus *Lactobacillus* including, *L. plantarum* (de Las Rivas et al. 2006; Tanganurat et al. 2009), *L. casei* (Cai et al. 2007), *L. sanfranciscensis*

(Picozzi et al. 2010), *L. paracasei* (Parolo et al. 2011), *L. salivarius* (Raftis et al. 2011), *L. delbrueckii* (Tanigawa and Watanabe 2011), *L. johnsonii* (Buhnik-Rosenblau et al. 2012), *L. sakei* (Chaillou et al. 2013) and *L. acidophilus* (Ramachandran et al. 2013).

1.4.6.1 *Lactobacillus acidophilus* Complex

In 2013, Ramachandran et al. analysed a subset of 52 isolates in the *L. acidophilus* complex (*L. acidophilus* 5 strains, *L. amylovorus* 11 strains, *L. crispatus* 11 strains, *L. gallinarum* 8 strains, *L. gasseri* 9 strains and *L. johnsonii* 8 strains) and genotyped them using a novel multilocus sequence typing (MLST) scheme, seven housekeeping genes, *fus*A, *gpm*A, *gyr*A, *gyr*B, *lep*A, *pyr*G and *rec*A (Ramachandran et al. 2013). Forty sequence types (STs) were identified that allowed the isolates to be successfully clustered within the six species. Sequence analysis resolved an average of 150 variable nucleotide sites per locus, ranging from 17 to 25, for the seven MLST genes. Analysis of the observed alleles suggested that nucleotide substitutions within five of the seven MLST loci had reached saturation, a finding that emphasised the highly diverse nature of the *L. acidophilus* complex and our unconventional application of a typically intraspecies molecular typing tool.

1.4.6.2 *Lactobacillus casei*

In 2007, Cai et al. reported a genotypic and phenotypic characterisation of *L. casei* isolates from different ecological niches, plant materials (9 isolates), human gastrointestinal tracts (7 isolates), human blood (1 isolate), cheeses from different geographical locations (22 isolates) and one isolate of unknown origin. These 40 isolates of *L. casei* were divided into three clusters: a cheese cluster, a silage cluster and a cluster that included isolates from a range of different origins (primarily the human GI tract and cheeses) (Cai et al. 2007). A high degree of recombination and a high level of phylogenetic heterogeneity were observed in the 40 *L. casei* strains. These events may have facilitated rapid adaptation of *L. casei* to different environments and was consistent with previous reports for other *Lactobacillus* species that also displayed a recombinatorial population structure (de Las Rivas et al. 2006). For example, strong evidence for intraspecies recombination was observed in *L. plantarum* in the form of both the presence of network structures in split decomposition analysis and linkage equilibrium (de Las Rivas et al. 2006). More interestingly, a high *d*S/*d*N ratio was observed for six MLST genes which is suggestive of strong purifying selective pressure. The majority of synonymous SNPs were preferentially associated with elimination of variations in amino acids. Some of the non-synonymous SNPs may lead to adaptive niche expansion and provide a selective advantage for *L. casei* with respect to survival in nonconventional habitats. Additionally, based on the 199 SNPs found in the study,

it was estimated that the major lineages of *Lb. casei* diverged approximately 1.5 million years ago.

Subsequently, to gain a broader view of the genetic variability within *L. casei*, Cai et al. conducted a comparative genome hybridisation (CGH) analys is of a collection of 21 strains from a variety of environmental habitats (cheese, 7 strains; plant material, 8 strains; and human source, 6 strains) (Cai et al. 2009). This analysis resulted in the identification of 25 hypervariable regions. One of these regions contains an overrepresentation of genes involved in carbohydrate utilisation and transcriptional regulation and was thus proposed as a lifestyle adaptation island. Differences in the *L. casei* genome inventory revealed both gene gain and gene decay. Gene gain, via acquisition of genomic islands, is likely to confer a fitness benefit in specific habitats. Gene decay, that is, loss of unnecessary ancestral traits, was observed in the cheese-derived isolates and is likely to result in enhanced fitness in the dairy niche (Cai et al. 2009).

Diancourt et al. also reported that all 52 isolates of *L. casei* examined could be distinguished from 31 STs using the MLST scheme on the seven remaining genes, *fus*A, *ile*S, *lep*A, *leu*S, *pyr*G, *rec*A and *rec*G (Diancourt et al. 2007). The results showed that homologous recombination during diversification occurred in all 52 isolates examined, including a putative intragenic import of DNA into one strain. Nucleotides were estimated to change four times more frequently by recombination than by mutation. However, statistical congruence between individual gene trees was retained, indicating that recombination was not frequent enough to disrupt the phylogenetic signal.

In 2013, another MLST scheme was developed by a research team in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P. R. China (Sun et al. unpublished data) for *L. casei* and used to investigate the genetic polymorphisms and evolutionary relationships amongst isolates. A total of 230 *L. casei* isolates from naturally fermented yogurt sampled in Inner Mongolia and Tibet, Xinjiang, Sichuan and Gansu of China and Bulgan, Zavkhan, Ovorkhangai and Arkhangai Provinces of Mongolia, were genotyped using a novel multilocus sequence typing (MLST) scheme, ten housekeeping genes, *car*B, *phe*S, *uvr*C, *pyr*G, *rec*A, *rpo*C, *clp*X, *dna*A, *gro*EL and *mur*E. A total of 174 STs were clustered in 21 clonal complexes (CC) by combining the ten gene loci using the Bionumerics 6.0 and eBURST (Fig. 1.7). The results showed that the evolution of *L. casei* isolates was correlated with geographic environment, such as climate and altitude.

1.4.6.3 *Lactobacillus delbrueckii*

Genetic diversity within *L. delbrueckii* subsp. *delbrueckii*, subsp. *bulgaricus*, *L. delbrueckii* subsp. *indicus*, *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *sunkii* using an MLST with seven housekeeping genes scheme, *fus*A, *gyr*B, *hsp*60, *ile*S, *pyr*G, *rec*A and *rec*G (Tanigawa and Watanabe 2011). The results showed

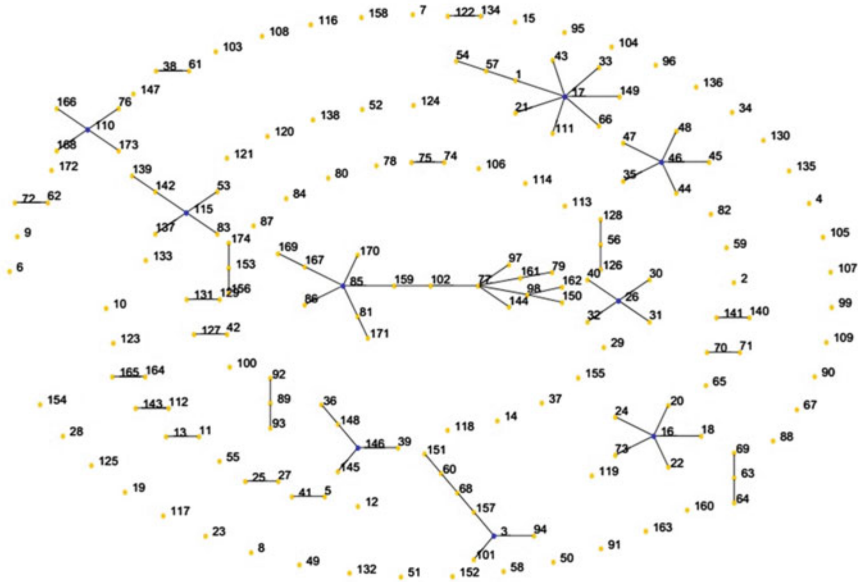


Fig. 1.7 Population snapshot of 230 *L. casei* isolates by eBURST (Sun et al. unpublished data)

that the 41 isolates of *L. delbrueckii* examined were divided into 34 STs, and 8 groups. These groups could be discriminated as representing different subspecies. The results of synonymous and non-synonymous changes in the allele sequences showed that dN/dS ratios were much lower than 1 (0.0051 to 0.0096), which implied purifying selective pressure (negative selection). In contrast, the nucleotide diversity values of *L. delbrueckii* were a little higher than those previously reported for *L. casei* which ranged from 0.0002 to 0.0076 (Diancourt et al. 2007) and nucleotide diversity values for *L. plantarum* which ranged from 0.0004 to 0.0072 (de Las Rivas et al. 2006).

In 2013, a total of 247 *L. delbrueckii* subsp. *bulgaricus* isolates were evaluated using MLST protocols by a research team in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P. R. China (Sun et al. unpublished data). The isolates were from naturally fermented yogurt sampled in Inner Mongolia, Xinjiang, Qinghai, Sichuan, Gansu, Yunan and Tibet of China, Gobi Altai, Sukhbaatar, Dornod, Khentii, Selenge, Orkhon, Bulgan, Khovsgol, Tov, Zavkhan, Ovorkhangai, Ulaanbaatar and Arkhangai of Mongolia. There were 115 STs distinguished that clustered into 14 CCs based on sequence analysis of ten gene loci. The concatenated sequence of the ten gene loci showed a network-like structure (Fig. 1.8), suggesting that the gene fragments had undergone widespread

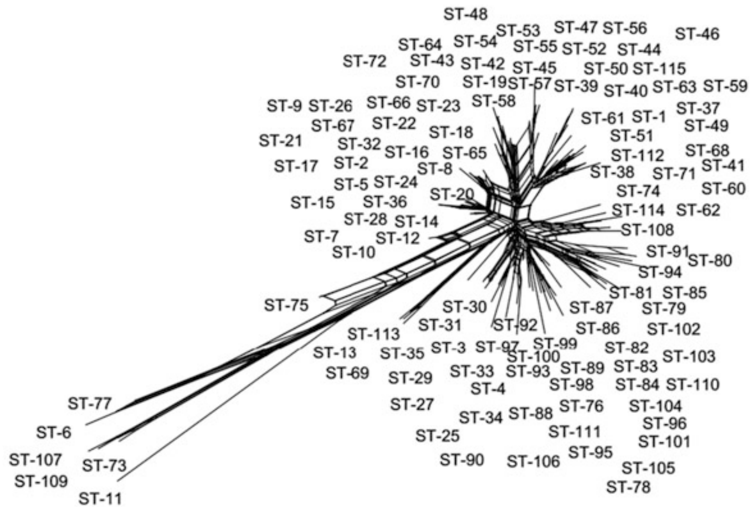


Fig. 1.8 Combined split decomposition of alleles for all 10 MLST loci (Sun et al. unpublished data)

associative recombination. For these reasons, the results suggest that recombination and selective pressure are likely to contribute to the evolution of *L. delbrueckii* subsp. *bulgaricus*.

1.4.6.4 *Lactobacillus johnsonii*

Genetic diversity of 47 isolates of *L. johnsonii* derived from various hosts has been reported by Buhnik-Rosenblau et al. (2012). The 47 strains *L. johnsonii* examined were divided into 28 STs that were distributed in three clear clusters. The clusters of genetic relationships amongst the strains indicated that each cluster of *L. johnsonii* strains was comprised of isolates from a different host, i.e. chickens, hum ans or mice. These results, suggest strong phylogenetic separation among *L. johnsonii* isolates in relation to host specificity. This could have arisen as a result of coevolution of the host and its GIT microbiota (Dethlefsen et al. 2007; Neish 2009; Ley et al. 2006). In fact, host-driven evolution has also been observed in another lactobacilli species, *L. reuteri* (Oh et al. 2010). According to the recently proposed 'hologenome theory' (Zilber-Rosenberg and Rosenberg 2008), the host and its symbiotic microbiota are one unit of selection in evolution. Indeed, previous analysis of the *L. johnsonii* genome showed the absence of genes required for several metabolic pathways (Pridmore et al. 2004) emphasising the high dependence of *L. johnsonii* on its host and further supports the concept that *L. johnsonii* and its host are one evolutionary unit of selection. Since chickens, humans and mice are distinct genetic species divided during

evolution, *L. johnsonii* strains associated with them may be evolutionary separated as distinct holobionts.

1.4.6.5 *Lactobacillus paracasei*

Genetic diversity of 75 strains of *L. paracasei* isolated from dental biofilms has been analysed using MLST scheme (Parolo et al. 2011). Fourteen STs were identified based on seven housekeeping genes *fus*A, *ile*S, *lep*A, *leu*S, *pyr*G, *rec*A and *rec*G. Phylogenetic analysis of the MLST data by split decomposition analysis indicated intraspecies recombination in the *L. paracasei* populations. This was consistent with the homologous recombination observed previously during diversification of *L. casei* clones (Cai et al. 2007; Diancourt et al. 2007). However, unlike *L. casei* the recombination was not related to niche specificity which may be because *L. paracasei* has an exogenous origin.

1.4.6.6 *Lactobacillus plantarum*

Tanganura et al. analysed a 16 strains of *L. plantarum* using an MLSA scheme of six housekeeping genes, *pgm*, *ddl*, *gyr*B, *pur*K1, *gdh* and *mut*S (Tanganurat et al. 2009). These strains were divided into 14 STs, with 12 of them represented by only one strain. Compared to16S rRNA sequence analysis and restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified 16S–23S rDNA intergenic spacer region, MLST was able to characterise *L. plantarum* isolates more precisely and unambiguously. In additional, split decomposition analysis of *L. plantarum* indicated that recombination played a role in creating genetic heterogeneity (Tanganurat et al. 2009).

In 2013, another MLST scheme was developed for *L. plantarum* by a research team in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P. R. China and used to investigate genetic polymorphisms and evolutionary relationships amongst isolates (Sun et al. unpublished data). A total of 181 *L. plantarum* isolates were investigated from naturally fermented yogurt sampled in Inner Mongolia, Xinjiang, Qinghai, Sichuan, Gansu and Tibet of China, Dornogvi and Ulaanbaatar of Mongolia. These isolates were divided into 72 STs, and clustered ten CC groups, based on eight housekeeping genes, *phe*S, *uvr*C, *pyr*G, *rec*A, *clp*X, *mur*C, *gro*EL and *mur*E. The largest CC, represented by 22 STs, contained most of isolates from Sichuan and Inner Mongolia (Fig. 1.9). The other two CCs were represented by STs of isolates from Mongolia and Xinjiang Provinces, respectively. Minimum-spanning tree analyses showed that evolution of *L. plantarum* isolates was correlated with climate and altitude.

1.5 The Genus *Lactococcus*

1.5.1 History

Lactococci are coccoid Gram-positive, catalase-negative, nonmotile and facultative anaerobic bacteria, with L-(+)-lactic acid as their predominant end product of glucose fermentation. They are commonly called mesophilic lactic streptococci and most strains react with group N antisera (Lancefield 1933). Lactococci have complex and variable nutritional requirements, and they typically inhabit plants, animals and their products.

It is tempting to suggest that the first isolation, identification and description of the chemical entity lactic acid from sour milk in Sweden by Carl Wilhelm Scheele in 1780 was actually L-lactic acid produced by lactococci. Nevertheless, in 1873, Joseph Lister first obtained and described the first pure bacterial culture of *Lactococcus lactis*, which was termed bacterium lactis. Lister studied lactococci in an attempt to prove Pasteur's germ theory of fermentative changes. Currently, the genus *Lactococcus* comprises 11 recognised species and subspecies (http://www.bacterio.net/lactococcus.html, 20 December 2013): *Lac. chungangensis*, *Lac. fujiensis*, *Lac. garvieae*, *Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *hordniae*, *Lac. lactis* subsp. *lactis*, *Lac. lactis* subsp. *tructae*, *Lac. piscium*, *Lac. plantarum*, *Lac. raffinolactis* and *Lac. taiwanensis* (Schleifer et al. 1985; Cai et al. 2011; Cho et al. 2008; Pérez et al. 2011; Williams et al. 1990; Chen et al. 2012).

DNA G+C content (mol.%): 34–43
Type species: *Lactococcus lactis* (Lister 1873) Schleifer et al. 1986

1.5.2 Cell Morphology and Cultural Characteristics

The common morphology of lactococci consists of 0.5- to 1-μm diameter spheres or ovoid cells that exist in pairs or chains. Cells of lactococci often elongate in the direction of the chain, which makes them difficult to differentiate from lactobacilli. Lactococcal cultures usually grow in the range 10–40 °C, although some species may grow under temperatures as low as 7 °C upon prolonged incubation for 10–14 days (Sakala et al. 2002a). Cultures typically grow in 4.0 % (w/v) NaCl; however, *Lac. lactis* subsp. *cremoris* tolerates only 2.0 % NaCl, which is the only known exception. Lactococci grow best at near-neutral pH values in media but cease to grow at about pH 4.5.

Lactococci are homofermentative microaerophilic bacteria, and their growth is not severely affected by aeration. However, highly toxic oxygen compounds such as superoxide, hydrogen peroxide and hydroxyl radicals are generated by *Lac. lactis* under aerobic conditions. Lactococci are catalase negative, but they have NADH oxidases and superoxide dismutases, which are upregulated under aerobic

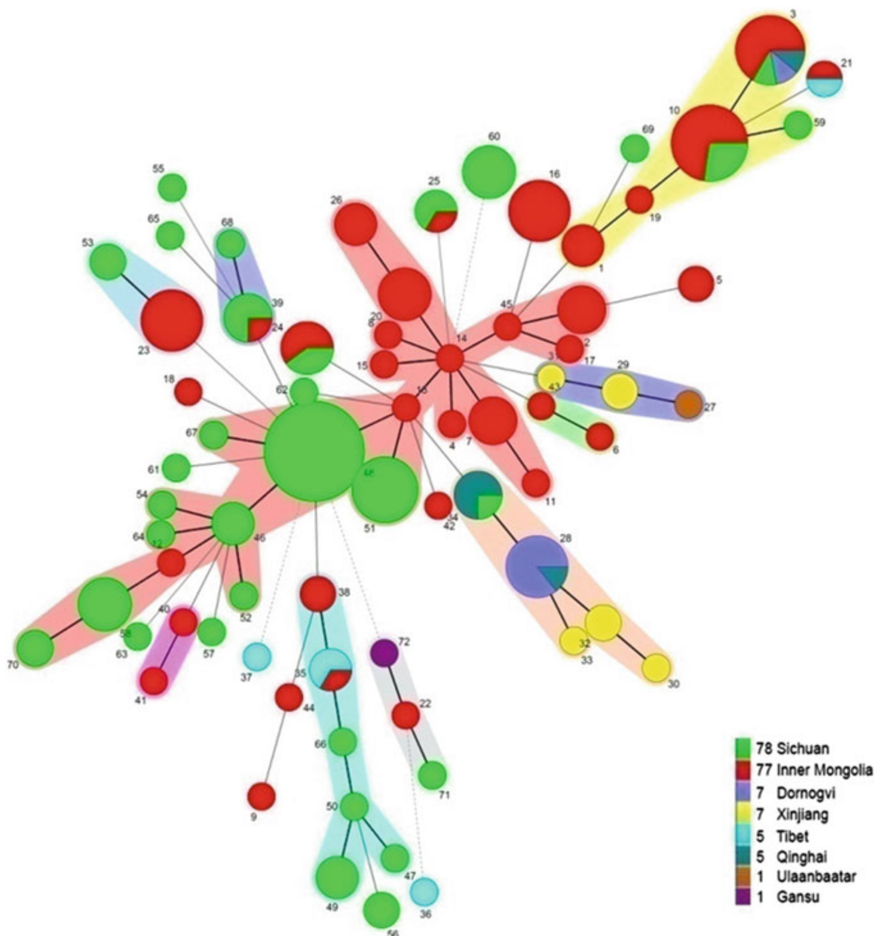


Fig. 1.9 Minimum spanning tree analysis of the 181 isolates *L. plantarum* based on allelic profiles at the eight genes. Each circle corresponds to a ST, and the circle size denotes the number of strains sharing the same ST. Coloured zones between some groups of circles indicate that these profiles belong to the same clonal complex. (Sun et al. unpublished data)

stress conditions (Häggström 1984). In the presence of a heme source in a complex medium, *Lac. lactis* exhibits respiration via a process that utilises its terminal cytochrome oxidase (*bd*) (Gaudu et al. 2002). This property leads to increased survival and growth yield, decreased lactate production and increased acetate and diacetyl in the medium. Therefore, lactococci may be in transition from using a fermentative to an oxidative growth strategy.

1.5.3 Phylogenetic Position and Genomes

The phylogenetic position of lactococci within *Firmicutes* was established by comparison of the 16S rRNA gene sequences (Schleifer and Ludwig 1995a); along with *Streptococcus* and *Lactovum*, the *Lactococcus* genus is a member of the family *Streptococaceae*, in the order *Lactobacillales*. The *Lactococcus* genus was transferred from the Lancefield group of N streptococci (Schleifer et al. 1985). Therefore, a close phylogenetic relationship exists among the genera *Lactococcus*, *Streptococcus* and *Enterococcus*.

The size range of complete lactococci genomes is ~1.95 (Morita et al. 2011b) to 2.64 (Siezen et al. 2010) Mbp. Currently, more than 10 complete genomes of lactococci are available, covering different strains of three species and subspecies, which include *Lac. garvieae*, *Lac. lactis* subsp. *cremoris* and *Lac. lactis* subsp. *lactis*. The genetic study of these bacteria led to two important milestones: the discovery of extrachromosomal elements (plasmids) in dairy lactococci and the elucidation of the complete genome sequence of *Lac. lactis* subsp. *lactic* IL1403 (Bolotin et al. 1999, 2001).

1.5.4 Taxonomy, Phylogeney and Evolution

The first lactococci identified (*Lac. Lactis*) by Joseph Lister in 1873 was later renamed *Streptococcus lactic* (Löhnis 1909). On the basis of exhaustive reinvestigation including cell wall analysis, biochemical properties and DNA–DNA hybridisation, Schleifer et al. proposed in 1985 the separate classification of N streptococcus (Lancefield 1933) from the oral streptococci, the enterococci and the hemolytic streptococci, using the new genus name *Lactococcus*. The genus *Lactococcus* was originally described based on the morphological, physiological and biochemical characteristics; however, the *Streptococcus, Enterococcus* and *Leuconostoc* also form cocci that occur as chains or pairs, and it is difficult to distinguish these genera from *Lactococcus* on a morphological basis (Wijtzes et al. 1997). Molecular approaches have been used subsequently to detect and differentiate new species and infer phylogenetic relationships among the lactococci.

In the last two decades, molecular methods—particularly 16S rRNA gene sequencing—have facilitated identification of diverse bacteria. The 16S rRNA gene sequencing technique is the gold standard for categorising an unknown, newly isolated strain to a species. Because of its molecular clock properties and the large database for sequence comparison, it has served as a powerful tool for identifying phylogenetic relationships among bacteria (Woese 1987). Figure 1.10 depicts a phylogenetic tree of the 16S rRNA gene sequences currently available for the species included in the genus *Lactococcus*. In addition, some studies have used 16S rRNA gene sequences to construct genus- and species-specific nucleotide probes (fluorescent or radioactively labelled) for the identification and differentiation of lactococci in situ and in single colonies (Betzl et al. 1990; Salama et al. 1991).

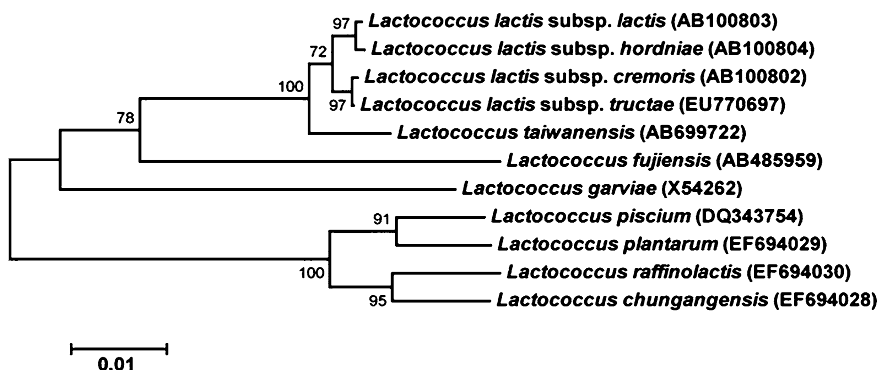


Fig. 1.10 Phylogenetic relationships between species in the genus *Lactococcus* based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method

However, the 16S rRNA gene sequence is too conserved to provide sufficient resolution at the species and subspecies levels (Weisburg et al. 1991; Stackebrandt and Goebel 1994). The *Lac. lactis* subsp. *tructae* type strains showed 99.4 % 16S rRNA gene sequence similarity to that of the *Lac. lactis* subsp. *lactis* ATCC 19435^{T} and *Lac. lactis* subsp. *hordniae* ATCC 29071^{T} type strains; in addition, it showed 99.9 % similarity to the *Lac. lactis* subsp. *cremoris* ATCC 19257^{T} (Pérez et al. 2011). The 16S rRNA gene analysis of closely related strains might show inaccuracies and inconsistencies with the results obtained by other methods.

DNA–DNA hybridisation studies represented a critical milestone for the clustering of the genus *Lactococcus* and its species and subspecies (Schleifer et al. 1985). This method provides higher resolution than the 16S rRNA gene sequencing method, and the 70 % criterion has been a cornerstone for describing bacterial species (Cho and Tiedje 2001). The DNA–DNA similarity is ~15–20 % among *Lactococcus* species, whereas the similarity is more than 60 % within *Lac. lactis* subspecies (*Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *hordniae*, *Lac. lactis* subsp. *lactis* and *Lac. lactis* subsp. *tructae*) (Pérez et al. 2011). However, this method has limitations due to disadvantages such as the laborious nature of pairwise cross-hybridisations, the requirement for isotope use and the difficulty in establishing a central database.

DNA fingerprinting technologies, including restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) have been useful for the molecular typing and discrimination of closely related lactococci species. Köhler et al. investigated a PCR–RFLP technique that specifically differentiated two subspecies *Lac. lactis* subsp. *cremoris* and *Lac. lactis* subsp. *lactis*. This technique facilitated the successful screening and grouping of new lactococcal isolates. It is critical that the techniques used to differentiate between *Lac. lactis* subspecies allow for the correlation between phenotypic and genotypic identification (Köhler et al. 1991). Deveau and Moineau

used a modified RFLP procedure for rapid characterisation of *Lac. lactis* strains that produce exopolysaccharides (EPS). The authors inferred that the availability of such an effective cataloguing system would facilitate the identification of lactococcal strains that produce novel EPS (Deveau and Moineau 2003). In 2003, Ravelo et al. used the RAPD technique to evaluate the genetic diversity in *Lac. garvieae*, an important pathogen of fish (Ravelo et al. 2003). The authors separated 57 *Lac. garvieae* with different hosts and geographical origins into three genetic groups: the Spanish, Portuguese, English and Turkish strains (group A), the Italian and French strains (group B) and the Japanese strains (group C). This study indicated that RAPD profiling constitutes a useful tool for epidemiological studies of this fish pathogen. Tailliez et al. applied the RAPD technique to resolve previous contradictions in lactococcal classifications (Tailliez et al. 1998). Three primers were used to classify 113 strains and integrate the resulting information using computer software. An unusual taxonomic structure was revealed within *Lac. lactis*, which comprised three major RAPD groups: two distinct genetic groups of strains showed indistinguishable phenotypes, while two phenotypically distinct groups were genetically homologous. Therefore, the authors hypothesised that a subfamily of the subsp. *lactis* group gave rise to the *cremoris* subspecies. In 2007, Foschino et al. investigated the genetic relatedness between *Lac. garvieae* strains isolated from fish and dairy samples collected in northern Italy, using RAPD, *Sau*-polymerase chain reaction (PCR) and AFLP (Foschino et al. 2008). RAPD fingerprinting proved to be a molecular tool for comparing isolates, whereas *Sau*-PCR and AFLP analyses were useful techniques for investigating the distribution of *Lac. garvieae* populations in the environment. Tanigawa et al. discriminated species or subspecies in the genus *Lactococcus* by proteomic identification, and well as phenotypic and genotypic identification. The genotypic identification included 16S rRNA and *rec*A gene sequences and AFLP. This study was the first to use the AFLP method to discriminate *Lac. lactis* strains (Tanigawa et al. 2010). Subsequently, Kutahya et al. developed a high-resolution AFLP methodology to achieve the delineation of closely related *Lac. lactis* strains (Kutahya et al. 2011). Tanskanen determined the PFGE pattern of *Sma*I digests of 29 strains of *Lac. lactis* subsp. *lactis* and subsp. *cremoris.* The results demonstrated that this technique is a simple and reliable method for identifying lactococcal strains and investigating their evolution (Tanskanen et al. 1990). In 1998, Kelly et al. used the PFGE technique to characterise the lactococci isolated from minimally processed fresh fruit and vegetables. However, little information regarding the population structure and gene diversity/evolution of the species was obtained (Kelly et al. 1998).

The usefulness of housekeeping genes in bacterial taxonomy and phylogeny has been reported for several genera of lactic acid bacteria (Maiden 2006), including Gram-positive cocci of the genus *Streptococcus*, a genus that is phylogenetically close to *Lactococcus* (Glazunova et al. 2009). For lactococci, the partial sequences of the *recA* (recombinase A) and *rpo*B (β subunit of RNA polymerase) gene sequences in combination with 16S rRNA gene sequences have been used effectively to identify new species or subspecies. In 2010, the fourth subspecies within

Lac. lactis, *Lac. lactis* subsp. *hordniae*, was isolated from the intestinal mucus of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) (Pérez et al. 2011). In this study, the partial sequences of two housekeeping genes, *recA* and *rpo*B, were obtained from type strains of genus *Lactococcus*, and the results showed that the *rpo*B and *rec*A genes could be useful for differentiation of species within the genus *Lactococcus* (Wang et al. 2009). Using an approach called multilocus sequence typing (MLST), researchers performed DNA sequence analysis in several housekeeping genes or other protein-coding gene sequences to discriminate closely related species and analyse the genetic diversity and phylogenetic relationships. This method was shown to be a powerful technique for bacterial typing (Enright and Spratt 1999), providing critical information for evolutionary history, population structure and long-term epidemiology of bacterial species (Maiden et al. 1998). Although MLST has been used principally to study major bacterial pathogens, several recent MLST schemes have been developed for lactic acid bacteria (LAB) species.

Lactococcus lactis, an important species of the genus *Lactococcus* used in dairy starter cultures, comprises four subspecies: *Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *lactis*, *Lac. lactis* subsp. *hordniae* and *Lac. lactis* subsp. *tructae*. Among them, *Lac. lactis* subsp. *lactis* is found in a variety of environments, including animal sources, dairy products and plant surfaces (Klijn et al. 1995; Nomura et al. 2006), whereas the subspecies *Lac. lactis* subsp. *cremoris* is isolated primarily from raw milk and other dairy products (Urbach et al. 1997). These two subspecies are used widely for industry and research and play a key role in determining shelf-life, preservation and organoleptic quality, thereby influencing the quality and safety of these fermented products (Smit et al. 2005). Although *Lac. lactis* subsp. *lactis* and subsp. *cremoris* differ by no more than 0.7 % in their 16S rRNA gene sequences (Salama et al. 1991), they display an average of only 85 % DNA identity at the genomic level (Wegmann et al. 2007). Consequently, it has been difficult to accurately distinguish the two subspecies.

In 2007, Rademaker et al. analysed a subset of 89 strains of *Lac. lactis* subsp. *cremoris* and *Lac. lactis* subsp. *lactis* isolates using a novel multilocus sequence analysis (MLSA) scheme (Rademaker et al. 2007). Six partial genes (*atpA*, encoding ATP synthase alpha subunit; *pheS*, encoding phenylalanine tRNA synthetase; *rpoA*, encoding RNA polymerase alpha chain; *bcaT*, encoding branched chain amino acid aminotransferase; *pepN*, encoding aminopeptidase N; and *pepX*, encoding X-prolyl dipeptidyl peptidase) revealed 363 polymorphic sites (total length, 1,970 bases) among 89 *Lac. lactis* isolates, with unique sequence types for most isolates. This technique allowed high-resolution cluster analysis in which dairy isolates formed subclusters of limited diversity within the genomic lineages. To contribute to the characterisation of the natural variability of *Lac. lactis*, Passerini et al. reported a comparative evaluation of the genetic and genomic diversity in a collection of 36 strains isolated from different ecological sources and geographical areas using a new MLST scheme and PFGE technology (Passerini et al. 2010). Six loci (*gly*A, *pgk*, *pdp*, *bca*T, *pep*XP and *rec*N) were chosen from 33 loci for the new MLST scheme. A total of 25 sequent types (STs) in 36 strains were distributed among 14 unique

singletons and two clonal complexes (CCs). After eBURST analysis, a good correspondence was observed between the strain origin and ST clustering. The MLST analysis revealed that *Lac. lactis* subsp. *lactis* was essentially clonal with infrequent intra and intergenic recombinations. In addition, despite its taxonomical classification as a subspecies, it displayed a genetic diversity as substantial as that within several other bacterial species. The results in this study also demonstrated that the use of MLST could facilitate the ecological and phylogenetic identifications of new lactococcal strains, and may be more informative than other genotyping methods. In 2013, an MLST scheme was developed for *Lac. lactis*, and the genetic polymorphisms and evolutionary relationships among strains in this species were investigated by a research team in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education in China (Xu et al. 2013).

A total of 197 *Lac. lactis* strains were isolated from naturally fermented yogurt made by Mongolians in Chifeng, Xilin Gol, Hulunbeir, Bayan Nur of Inner Mongolia, by Bai nationalist in Dali of YunNan, and by Tibetans in Qinghai, Szechwan, Gansu and Tibet of China from 2005 to 2009. A total of 12 housekeeping genes (*dna*A, *pyr*G, *rpo*B, *gro*EL, *rec*A, *clp*X, *car*B, *mur*C, *pep*N, *pep*X, *mur*E and *phe*S) were used for the MLST analysis. The 12 gene loci were combined using the Bionumerics 6.0 software, and MLST identified 72 different STs.

The method of split decomposition (Huson 1998) was used to assess the degree of tree-like structures present in the alleles at each locus in all 197 bacterial isolates. Split graphs for each locus showed tree-like or slight network-like structures, indicating that most of the genes were affected by intragenic recombination (Fig. 1.11a). The concatenated sequence of the 12 MLST genes showed a network-like structure (Fig. 1.11b), suggesting that overall the 12 gene fragments underwent widespread associative recombination. Therefore, the data suggested that recombination and selective pressures likely contributed to the evolution of *Lac. lactis*. The d_N/d_S ratios for 12 loci were <1, indicating a strong purifying selection against changes in amino acid composition.

To explore the evolutionary relationships among the 197 strains, eBURST analysis was used for assignment of STs to CCs. The results revealed that the 72 STs were divided among 14 CCs (CC1 to CC14) and 23 singletons (Fig. 1.12). Strains within CCs represented 88.3 % of the 197 strains. In Fig. 1.12, CC1 was the most common CC, represented by 13 STs, and ST5 was most prevalent among the 197 strains, seen in 38 (19.28 %) strains. ST5 had seven single locus variants (SLVs), ST18 had six SLVs and two double-locus variants (DLVs). All other CCs comprised only 2–5 STs and a limited number of strains.

Twelve loci sequences were concatenated, obtaining a much longer sequence alignment of 6,192 bp. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram of the concatenated sequence demonstrated the genetic relatedness among the *Lac. lactis* strains investigated in this study (Fig. 1.13). The eBURST and UPMGA tree analyses showed that the evolution of *Lac. lactis* isolates was correlated with geographic parameters, such as climate and altitude.

In addition, isolates of ST56 and ST70 were assigned to *Lac. lactis* subsp. *cremoris* based on UPMGA tree analysis, which were classified as *Lac. lactis*

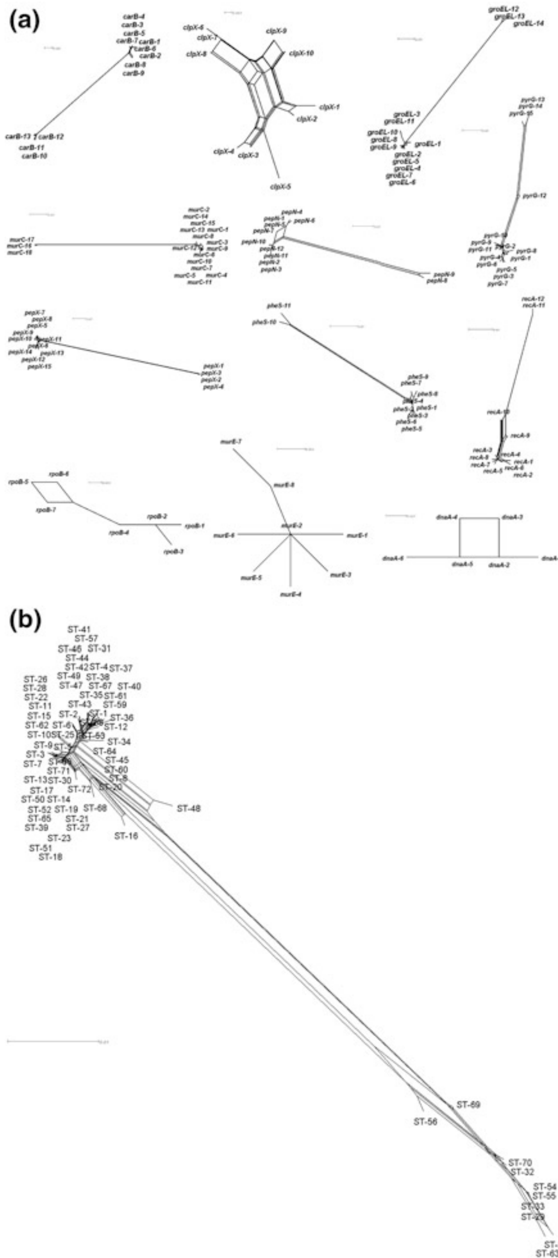


Fig. 1.11 Split decomposition analysis based on the allelic profiles of the *Lactococcus lactis* strains. **a** Split decomposition of alleles for individual multilocus sequence typing (MLST) loci and **b** combined split decomposition of alleles for all 12 MLST loci

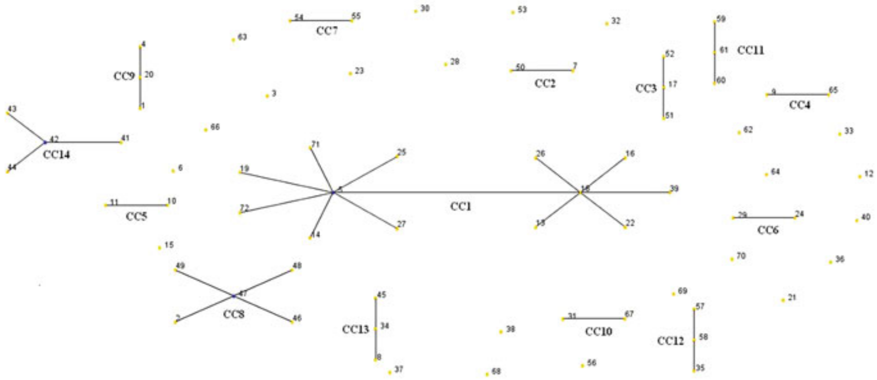


Fig. 1.12 eBURST analysis map of 197 *Lactococcus lactis* strains based on the allelic profiles of the 12 genes *car*B, *clp*X, *dna*A, *gro*EL, *mur*C, *mur*E, *pep*N, *phe*S, *pep*X, *pyr*G, *rec*A and *rpo*B

subsp. *lactis* according to the 16S rRNA gene sequence. Furthermore, the ST28 strain was found in *Lac. lactis* subsp. *cremoris*, which clustered in the *Lac. lactis*. subsp. *lactis* group. This provides strong evidence for reclassification of *Lac. lactis* subspecies by MLST, consistent with previous reports supporting MLST as a new method for distinguishing subspecies (Rademaker et al. 2007).

1.5.5 Conclusion

At present, the genus *Lactococcus* comprises 11 recognised species and subspecies. *Lactococcus lactis* is considered the most important industrial dairy starter microorganism and has been used for hundreds of years. Therefore, a large number of studies have focused on the taxonomy, phylogenesis and evolution of *lactococcal* species. In recent years, several molecular methods, including 16S rRNA gene sequencing, DNA fingerprinting technologies, protein-encoding gene sequencing and MLST, have been used widely to clarify *Lactococcus* phylogeny and to identify new species. Although no single classification system is perfect, MLST is an alternative high-resolution technique that is informative for understanding the genomic evolution and differentiation of *Lac. lactis*.

1.6 Genus *Leuconostoc*

1.6.1 Phylogenetic Status

Members of genus *Leuconostoc* are Gram-stain positive, non-motile, asporogenous, facultative anaerobic, ellipsoidal to spherical cells, often elongated, and arranged in pairs or chains (Tanigawa and Watanabe 2011; Garvie 1984). *Leuconostoc* spp. are

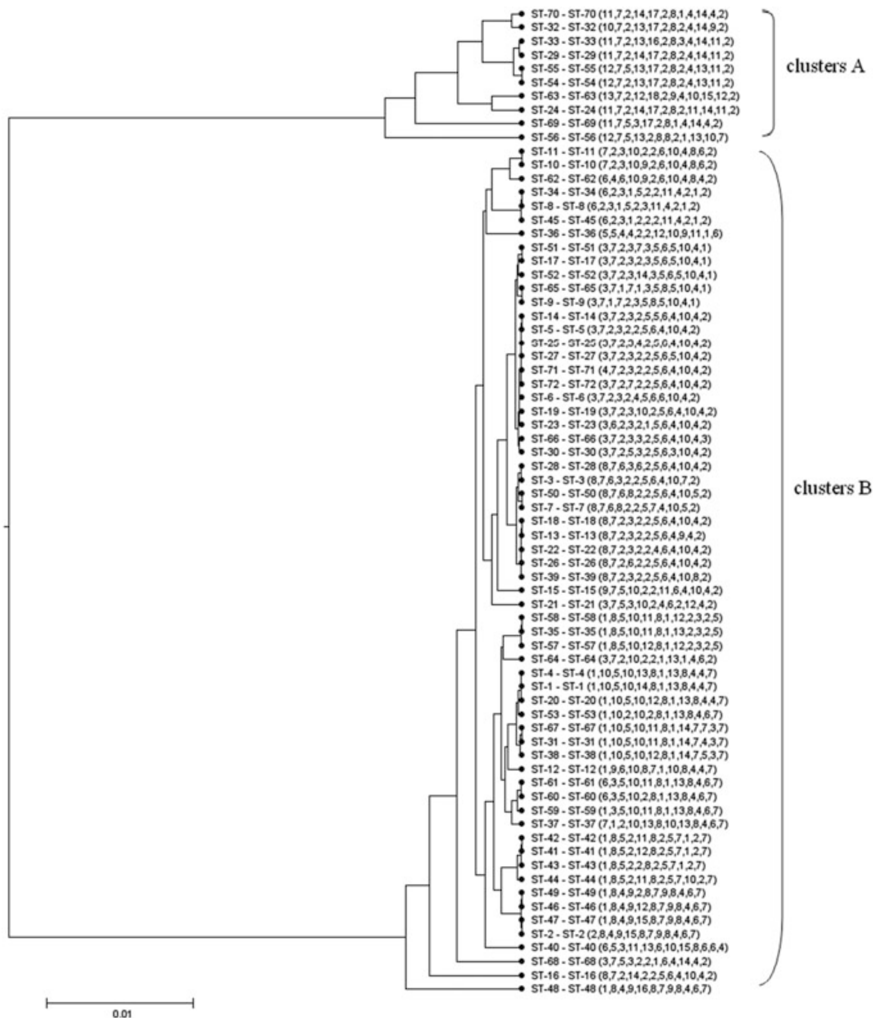


Fig. 1.13 An unweighted pair-group method with arithmetic mean (UPGMA) tree constructed from 72 concatenated nucleotide sequences of 12 MLST loci

environmental organisms often found in association with plant material, milk, dairy products, meat and other food products. These species are similar to heterofermentative *Lactobacilli*, especially gas-forming *Lb. confusus* and *Lb. viridescens* (Garvie 1984). *Leuconostoc* and *Lactobacillus* are often reported to be isolated from the same habitat and share many characteristics (Sharpe et al. 1972). Hucker and Pederson (1931) suggested that *Leuconostoc* spp. are intermediate forms between *streptococci* and *Lactobacilli* (Hucker and Pederson 1931).

Genus *Leuconostoc* comprises the following 13 recognised species: *Leuc. mesenteroides*, *Leuc. carnosum*, *Leuc. citreum*, *Leuc. fallax*, *Leuc. gasicomitatum*,

Leuc. *gelidum*, *Leuc*. *inhae*, *Leuc*. *kimchii*, *Leuc*. *lactis*, *Leuc*. *holzapfelii*, *Leuc*. *pseudomesenteroides*, *Leuc*. *palmae* and *Leuc*. *miyukkimchii* (Garvie 1984; Bjorkroth et al. 2000; Farrow et al. 1989; Kim et al. 2000; Kim et al. 2003; Martinez-Murcia and Collins 1991; Shaw and Harding 1989; Skerman et al. 1980). The first description of genus *Leuconostoc* was reported by Van Tieghem in 1878 (Friedland et al. 1990). In recent years, several species have been reclassified within genus *Leuconostoc*, and some new species have been added to the genus. In 1984, three species of *Leuc*. *mesenteroides* (*Leuc*. *mesenteroides* subsp. *mesenteroides*, *Leuc*. *mesenteroides* subsp. *dextranicum* and *Leuc*. *mesenteroides* subsp. *cremoris*) were reclassified as subspecies of *Leuc*. *mesenteroides* (Garvie 1984). *Leuc*. *fallax* is an atypical *Leuconostoc* isolate from sauerkraut, which was founded by Martinez–Murcia et al. in 1991; subsequently, a variety of *Leuc*. *fallax* strains were found in the heterofermentative stage of sauerkraut fermentation (Martinez-Murcia and Collins 1991). In 1993, the *Leuc*. *paramesenteroides* group of species was reclassified to a new genus called *Weisella* (Collins et al. 1993). In 1995, *Leuc*. *oenos* was reclassified to the genus *Oenococcus* as *O*. *oneni* (Dicks et al. 1995). In 2006, *Leuc*. *argentinum* was reclassified as a synonym of *Leuc*. *lactis*, after a numerical analysis of repetitive extragenic palindromic-PCR patterns, whole-cell protein profiles (SDS-PAGE) and fluorescent amplified fragment length polymorphism (FAFLP) band patterns (Vancanneyt et al. 2006). In 2008, four species of *Leuconostoc* (*Leuc*. *durionis*, *Leuc*. *ficulneum*, *Leuc*. *pseudoficulneum* and *Leuc*. *fructosum*) were assigned to a novel genus called *Fructobacillus* (Endo and Okada 2008). Subsequently, some novel strain, including *Leuc. holzapfelii*, *Leuc*. *palmae* and *Leuc*. *miyukkimchii*, were also identified from wine, kimchi, etc. (De Bruyne et al. 2007; Ehrmann et al. 2009; Lee et al. 2012b).

A phylogenetic tree was constructed through the neighbour-joining method, using the 16S rDNA sequence of 36 species of *Leuconostoc, Fructobacillus, Aerococcus, Carnobacterium, Vagococcus, Enterococcus, Lactococcus, Streptococcus, Lactobacillus* and *Weissella* deposited in GenBank (Fig. 1.14). Most *Leuconostoc* species used in phylogenetic tree construction are indicated in Table 1.3. The phylogenetic tree constructed revealed that the genus *Leuconostoc* (and related genera) were subdivided into two groups. The first group comprises the *Leuconostoc* group, three *Fructobacillus* species, three *Weissella* species and two *Lactobacillus* species. These strains are phylogenetically closely related and united into three subgroups. The first subgroup is the largest among the three, which comprise 16 *Leuconostoc* species and subspecies, *Leuc*. *mesenteroides*, *Leuc*. *pseudomesenteroides*, *Leuc*. *lactis*, *Leuc*. *kimchii*, *Leuc*. *citreum*, *Leuc*. *holzapfelii*, *Leuc*. *carnosum*, *Leuc*. *gasicomitatum*, *Leuc*. *gelidum*, *Leuc*. *inhae*, *Leuc*. *miyukkimchii, Leuc*. *palmae* and *Leuc*. *fallax* (http://www.bacterio.net/leuconostoc.html, 20 December 2013). Phylogenetically, *F*. *durionis*, *F*. *ficulneum* and *F*. *pseudoficulneum* are more closely related to 11 *Leuconostoc* species than to *Leuc*. *fallax*. Comparative 16S rRNA-sequencing analyses have clearly shown that 12 *Leuconostoc* species forms a distinct line of descent, which separates this species from other lactic acid bacteria. *Leuc*. *fallax* was distinct from the other *Leuconostoc* species, and was similar to those of other species of the genera

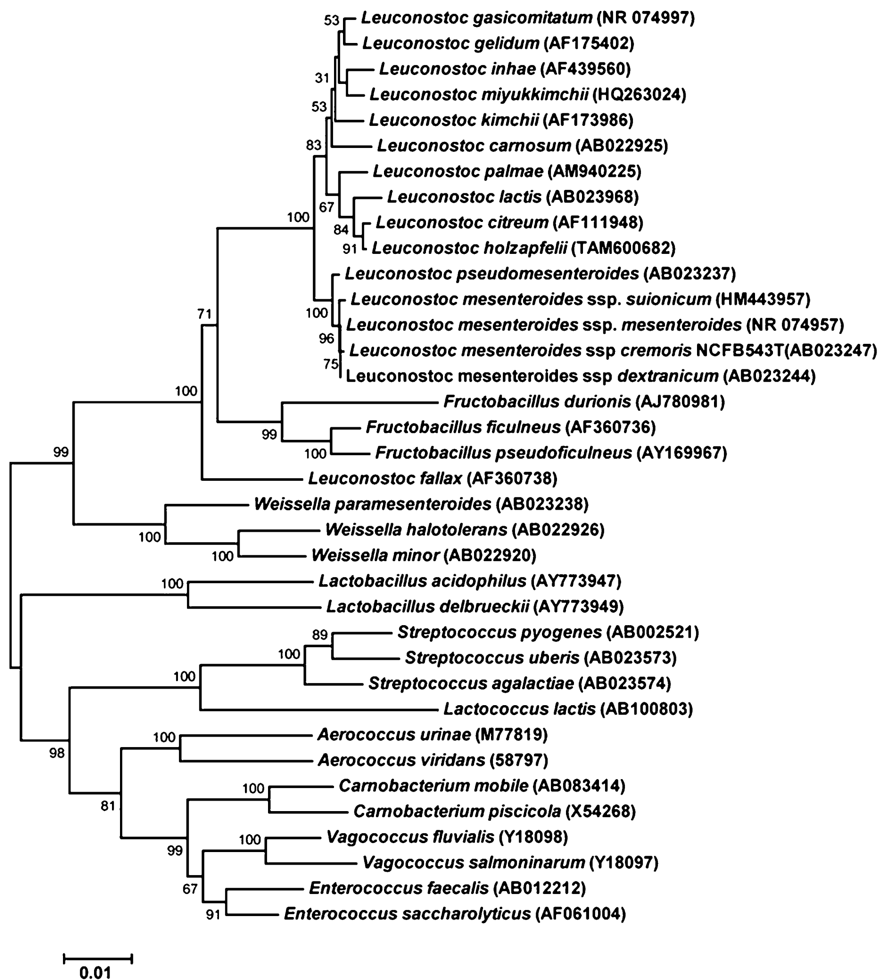


Fig. 1.14 Phylogenetic relationships of the genera *Leuconostoc* and lactic acid bacteria (and other related taxa) based on 16S rRNA gene sequences. The tree was constructed through the neighbour-joining method

Fructobacillus and *Weissella*. The second subgroup comprises three *Weissella* species (*W. paramesenteroides*, *W. halotolerans* and *W. minor*). The third subgroup comprises two *Lactobacillus* species (*Lb. delbrueckii* and *Lb. acidophilus*). The second group comprises two *Aerococcus* species, three *Streptococcus* species, two *Lactococcus* species, two *Carnobacterium* species, two *Vagococcus* species and two *Enterococcus* species. This analysis result indicated that these species in these genera are closely related.

Table 1.3 16S rRNA gene sequence information for Leuconostoc spp.

Species	Accession no.	Separating time	Source	References
Leuc. fallax	AF360738	1992	Sauerkraut	Martinez-Murcia and Collins (1991)
Leuc. carnosum	AB022925	1989	Chill-stored meats	Shaw and Harding (1989)
Leuc. gelidum	AF175402	1989	Chill-stored meats	Shaw and Harding (1989)
Leuc. mesenteroides	NR074957	1878	Unknown	Skerman et al. (1980)
Leuc. mesenteroides ssp. *cremoris*	AB023247	1929	Unknown	Garvie (1983)
Leuc. mesenteroides ssp. *dextranicum*	AB023244	1912	Unknown	Garvie (1983)
Leuc. mesenteroides ssp. *suionicum*	HM443957	2012	Unknown	Gu et al. (2012)
Leuc. miyukkimchii	HQ263024	2012	Kimchi	Lee et al. (2012b)
Leuc. pseudomesenteroides	AB023238	1967	Unknown	Garvie (1967)
Leuc. citreum	AF111948	1989	Human sources	Farrow et al. (1989)
Leuc. lactis	AB023968	1960	Unknown	Vancanneyt et al. (2006)
Leuc. inhae	AF439560	2003	Kimchi	Kim et al. (2003)
Leuc. kimchii	AF173986	2000	Kimchi	Kim et al. (2000)
Leuc. gasicomitatum	NR074997	2001	Unknown	Bjorkroth et al. (2000)
Leuc. holzapfelii	AM600682	2007	Coffee	fermentation
De Bruyne et al. (2007)				
Leuc. palmae	AM940225	2009	Palm wine	Ehrmann et al. (2009)

* Accession numbers and corresponding nucleotide sequences are obtained from the National Center for Biotechnology Information

1.6.2 *Current Molecule-Typing Methods Used for* Leuconostoc *spp.*

Typing methods for identifying different types of organisms within species are essential, epidemiological tools in infection prevention and control (Sabat et al. 2013). Typing methods are divided into two major categories, i.e. phenotypic and genotypic methods. Traditional phenotyping methods, such as serotype, biotype, phage-type or antibiogram, have been used for many years to isolate and characterise *Leuconostoc* and sometimes, to identify between species and sub-species. Compared with the phenotypic-typing methods, genotypic-typing methods also have some advantages, such as general applicability and high discriminatory power. Currently, several molecular-typing approaches have been attempted to analyse *Leuconostoc* spp.

Specific DNA probes, which are based on variable regions V1 and V3 of *Leuconostoc* 16S rRNA and amplified through PCR, are useful in identifying and

classifying *Leuconostoc* spp. (Klijn et al. 1991). Compared with classic methods, these probes are faster and more reliable in identifying strains. The DNA probe method requires very small amounts of bacterium DNA and can simultaneously identify a large number of strains. These probes are frequently used for characterisation of new isolates from nature.

Ribotyping was proven valuable in the identification of *Leuconostoc* spp., including *Leuc*. *carnosum* isolated from ham and processed meat, as well as *Leuc*. *gasicomitatum*, which is a spoilage-associated strain (Bjorkroth et al. 1998, 2000). Björkroth et al. (2000) used a polyphasic approach, including ribotyping and whole-cell protein analysis, for *Leuconostoc* spp. characterisation (Bjorkroth et al. 1998). Numerical analysis of total cellular proteins is generally considered to be useful for speciation of bacteria; and the authors have previously used ribotyping for *Leuc*. *carnosum* identification with good results (Bjorkroth et al. 2000). However, Villiani et al. (1997) considered that the technique was less reliable for identifying *Leuc*. *mesenteroides* (Villani et al. 1997). They found that *Leuc*. *mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum* showed the same ribopattern as that of *Leuc*. *lactis*, whereas *Leuc*. *mesenteroides* subsp. *cremoris* exhibited a pattern distinct from all the other species examined.

Randomly amplified polymorphic DNA (RAPD) typing has been frequently used for taxonomic and systematic analyses of various organisms (Bartish et al. 2000). *Leuc*. *fallax* strains isolated from sauerkraut fermentation were identified through a combination of internal transcribed spacer (ITS)-PCR analysis and sequencing of 16S rRNA gene variable region at the genus and species levels (Barrangou et al. 2002). Barrangou et al. (2002) proposed that the lack of molecular identification methods for *Leuc*. *fallax* could be responsible for historical failure to differentiate *Leuc*. *fallax* from *Leuc*. *mesenteroides* (Barrangou et al. 2002). Early reports demonstrated that the dominant strain in fermented rice cake was *Leuc*. *mesenteroides*; whereas later reports suggested that the dominant organism was *Leuc*. *fallax* (Cooke et al. 1987; Kelly et al. 1995). Pérez et al. (2002) developed a RAPD-PCR fingerprinting method to identify *Leuc*. *mesenteroides* strains isolated from tenerife cheese to its three subspecies (mesenteroides, cremoris and dextranicum) (Pérez et al. 2002). However, Cibik et al. 2000 suggested opposite opinions regarding the abovementioned results, and considered that the three subspecies of *Leuc*. *mesenteroides* might be biovars in the different molecular methods (Cibik et al. 2000). Simultaneously, (Cibik et al. 2000) utilised RAPD typing method to analyse the molecular diversity of a collection of 221 strains; these strains were identified as *Leuc*. *mesenteroides* or *Leuc*. *citreum* (Cibik et al. 2000).

Currently, repetitive-element PCR (Rep-PCR) method is widely used in bacterial classification and identification. Rep-PCR method is based on genomic fingerprint patterns to classify bacterial isolates (Sabat et al. 2013). According to Alegría et al. (2013), *Leuc*. *citreum*, *Leuc*. *mesenteroides* and *Leuc*. *lactis* were identified through the molecular methods; among these species, 22 strains were identified and typed using the Rep-PCR technique (Alegría et al. 2013).

Jang et al. (2003) developed a PCR-restriction fragment length polymorphism (RFLP) method to detect and identify the typical *Leuconostoc* spp. (Jang et al. 2003). In the present study, nine *Leuconostoc* species were identified, which did not contain the species *Leuc. fallax*.

The temporal temperature gradient gel electrophoresis (TTGE) technique was used to analyse differences in the 16S rDNA V3 regions of bacteria with low-G+C-content genomes (Ogier et al. 2008). Ogier et al. used the TTGE technique to separated different bacterial species that are present in several dairy products, including *Leuconostoc*. bacterial species in dairy samples were well distinguished by TTGE technique (Ogier et al. 2008).

Pulsed-field gel electrophoresis (PFGE) method was developed for separating and analysing long DNA fragments in alternating electric field. (Kelly et al. 1995) used this technique to differentiate among *Leuc. mesenteroides* subsp. *mesenteroides*, *Leuc. pseudomesenteroides*, *Leuc. citreum* and *Leuc. fallax* (Kelly et al. 1995). Kahala et al. (2008) also characterised *Leuconostoc* species using PFGE method at strain level (Kahala et al. 2008).

A multiplex PCR assay has been reported by (Lee et al. 2000) for identifying *Leuconostoc* spp., using species-specific primers targeted to the genes encoding 16S rRNA (Lee et al. 2000). This assay can exactly detect and differentiate *Leuconostoc* spp. within a few hours. This technique is proven a rapid and reliable method for *Leuconostoc* spp. differentiation from *Carnobacterium* specie, *Lactobacillus curvatus* and *Lactobacillus sakei* (Yost and Nattress 2000).

1.6.3 MLST Used for Leuconostoc spp.

Traditional and older molecular typing approaches for the characterisation of pathogenic microorganisms are lacking or have poor portability because the change of diversity index is difficult to compare among laboratories. To overcome these problems, the MLST methods have been invented. MLST provides a new approach to molecular epidemiology that can identify and track the global spread of virulent or antibiotic-resistant isolates of bacterial pathogens using the Internet (Enright and Spratt 1999). MLST is an extension of multilocus enzyme electrophoresis (MLEE), which relies on the differences in electrophoretic mobility of intracellular enzymes present in a bacterium. The MLST scheme, which was initially evaluated for *Neisseria meningitidis* in 1998, has been subsequently extended to other bacterial species, including *Streptococcus pneumoniae* (Enright and Spratt 1998), *Campylobacter jejuni* (Suerbaum et al. 2001), *Oenococcus oeni* (De Las Rivas et al. 2004), *Aspergillus fumigatus* (Bain et al. 2007), *Lactobacillus casei* (Cai et al. 2007), *Lactobacillus plantarum* (Tanganurat et al. 2009). Moreover, the scheme for *Yersinia pseudotuberculosis* (Laukkanen-Ninios et al. 2011), *Lactobacillus delbrueckii* (Tanigawa and Watanabe 2011) and *Staphylococcus aureus* are near publication (Saunders and Holmes 2014). To date, no MLST scheme has been developed for *Leuconostoc* spp.

In 2013, MLST schemes for *Leuconostoc* spp. (*Leuc. lactis* and *Leuc. mesenteroides*) have been developed in collaboration with the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P. R. China (Zhang et al. unpublished). These MLST schemes can be used for further studies on *Leuconostoc* spp. evolution and population.

1.6.3.1 The *Leuc. lactis* MLST Scheme

Fifty *Leuc. lactis* isolates, which are preserved in the Lactic Acid Bacteria Collection Center of the Inner Mongolia Agricultural University (LABCC), were examined and characterised. In the MLST scheme, DNA fragments (approximately ranging between 300 and 500 bp) of eight housekeeping genes are amplified and sequenced by PCR. For the housekeeping gene, unique sequence is assigned with a distinct allele number. In addition, based on the combination of eight allele numbers, a sequence type (ST) is provided. This ST profile was used to analyse the *Leuc. lactis* population structure. Fifty *Leuc. lactis* isolates used in this study were divided into 20 STs by combining the eight loci. ST14 type was the most common among 21 strains, followed by ST11 (four strains), ST3 (three strains), ST4 (three strains), ST1 (two strains), ST8 (two strains) and ST12 (two strains); the remaining 13 STs only have one strain.

Clonal complexes (CCs) are typically composed of a single predominant genotype with a number of much less common close relatives of this genotype (Feil et al. 2004). A CC contains genetically related, but not the same, organisms. To date, no standard definition of the CC is formulated. Indeed, *Leuc. lactis* isolates studied that share a minimum of six out of eight identical alleles with the central genotypes are assigned to CCs.

A large number of distinct STs are concluded in many MLST datasets, thereby providing an impression of infinite variability. For example, 50 *Leuc. lactis* strains were divided into 20 STs. However, these data investigation proved that while some STs are present at low frequency, other STs are more ubiquitous. The ubiquitous STs are usually isolated over the years and are located in geographically diverse regions. Furthermore, when the data are studied using heuristic methods (e.g. split decomposition or BURST algorithm), these ubiquitous STs inhabit a central location; wherein these STs have relatives that are derived from the ubiquitous STs themselves and exhibit a limited genetic diversity (Huson 1998; Enright et al. 2002). These findings form the basis of CCs to which they obtained their name; for example, the ST14 clonal complex and ST1 clonal complex of *Leuc. lactis* (Fig. 1.15).

Organisation into clonal complexes makes MLST data more suitable in reducing the potential of over-discrimination. For example, a collection of 50 *Leuc. lactis* strains of various sources included 20 STs that resolved into eight CCs. Among these CCs, 14 STs were clustered together to form two CCs and six singleton STs that cannot be assigned to any group as a single singleton. Figure 1.15 showed that the great majority of organisms of both the ST1 and the

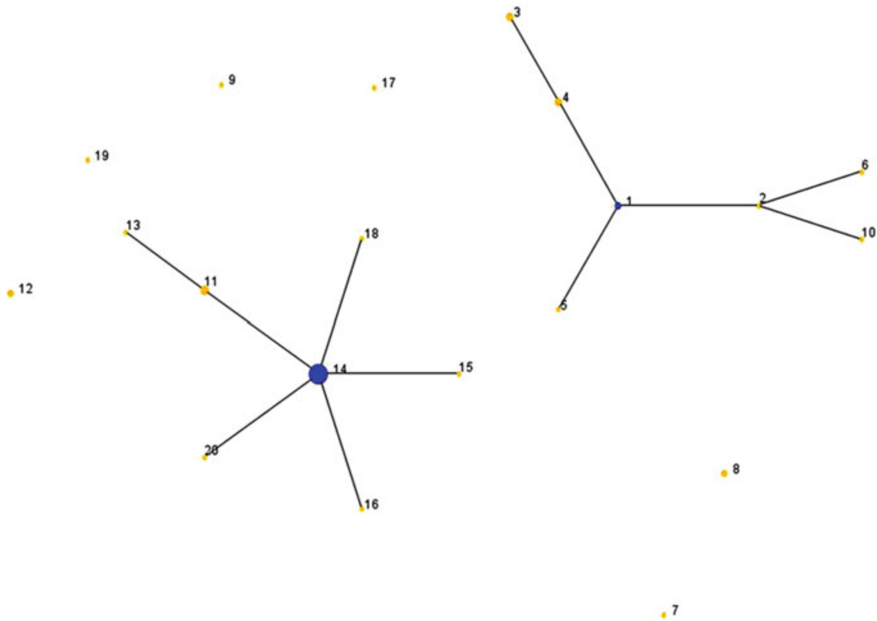


Fig. 1.15 Population snapshot of 50 *Leuc. lactis* isolates. In the eBURST diagram, two clonal complexes and six singletons are shown. ST1 and ST14 are the primary founders of two clonal complexes and most other isolates are single-locus variants (SLVs) of either ST1 or ST14

ST14 clonal complexes are SLVs (single-locus variants) of their respective, strongly supported primary founders. In some cases, limited diversity of the bacterial population can correctly assign isolates directly to clonal complexes, respectively (Feil et al. 2003).

The original proposition of MLST, in which the allele number was used as the primary unit of analysis, was suitable for organisms where horizontal transferred genes can occur frequently. To date, the sequence-type analysis and recombinant test (START) package software brings many preliminary analyses together that can be performed on MLST data (Jolley et al. 2001). For example, the split decomposition of the 20 sequence types from 50 *Leuc. lactis* isolates was conducted with SplitsTree and START 2.0 softwares in the MLST webset (http://eburst.mlst.net/) (Fig. 1.16).

To analyse data or to produce instructive graphical outputs by transforming evolutionary distances into a sum of weakly compatible splits with weights, Bandelt and Dress developed the split decomposition method (Bandelt and Dress 1992). Figure 1.16 is a visualisation of the phylogenetic distance dataset obtained by analysing eight MLST loci from *Leuc. lactis* isolates. This figure consists of parallelograms and individual edges. Considerable parallelogram-shape structures were found in the split graphs of the partial housekeeping genes and the combined alleles, which illustrate that recombination have occurred in some MLST loci of

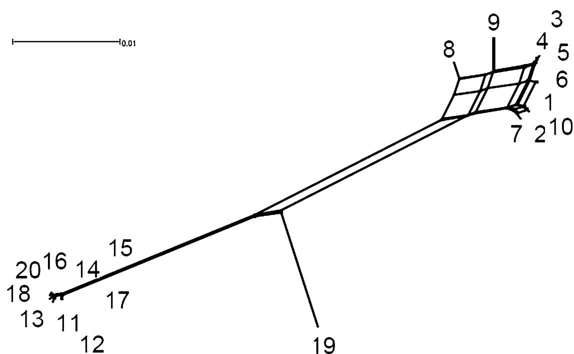


Fig. 1.16 Split decomposition analysis based on concatenated sequences of eight housekeeping genes of the 50 *Leu. lactis* strains. Multi-parallelogram formation indicated some recombination events. The numbering refers to ST

the *Leuc*. *lactis* strains. Evidences for the recombination occurs frequently in the genome sequence of *Leuconostoc* spp., because of the presence of the integration of horizontally acquired mobile elements; for example, bacteriophages, genomic islands and transposable elements (Meslier et al. 2012). In addition, some plasmids from *Leuconostoc* spp. were identified (Chang and Chang 2009; Jeong et al. 2007). In this split graph, we also found that 20 STs form two clusters that are separated from each other.

The original purpose of unweighted pair group method with arithmetic mean (UPGMA) was to construct taxonomic phenograms, which are trees that reflect the phenotypic similarities between operational taxonomic units (OTUs). Figure 1.17 shows an UPGMA dendrogram, which indicates the relatedness of 50 *Leuc*. *lactis* strains from various sources in Mongolia and the minority area of China. Two major clusters are evident—the larger group consisting of 34 strains and the other group of only 16 strains. Those strains of cluster A are closely related, which only differs in two out of the eight loci from the primary founder ST14; except a strain that belongs to ST19 which is a six-locus variant of the primary founder. Those strains of cluster B are distantly related, which differs in two or six out of the eight loci from the primary founder ST1.

1.6.3.2 The *Leuc. mesenteroides* MLST Scheme

Leuc. *mesenteroides* is the most commonly used heterofermentative dairy lactic acid bacteria. These strains are frequently used as dextran or bacteriocin producer in a number of fermented milk products and cheese types. A MLST scheme was developed for *Leuc*. *mesenteroides*. The system involved sequencing nine housekeeping genes and was applied to a panel of 136 isolates of *Leuc*. *mesenteroides* from various sources of Mongolia and minority area of China. Totally, 68 different sequence types were found among the 136 strains. Among these strains, ST 14 type was the most common with 13 strains.

The MLST data of the 136 *Leuc*. *mesenteroides* isolates were analysed by eBURST, with the stringent group definition of seven of nine shared alleles. Figure 1.18 is the eBURST diagram of the *Leuc*. *mesenteroides* isolates, which

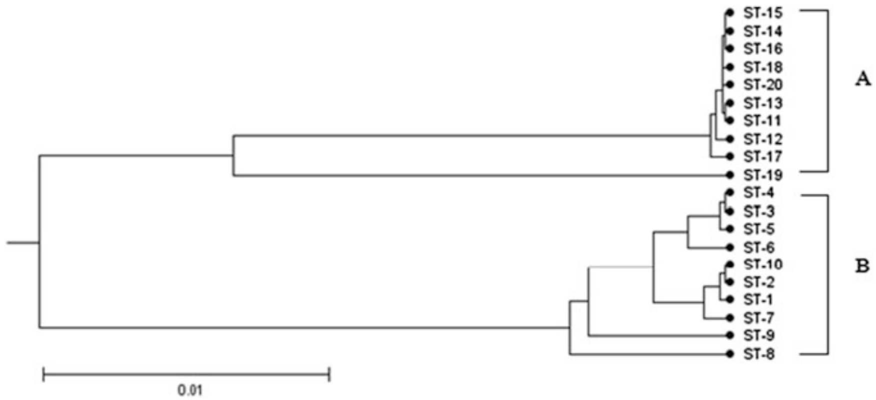


Fig. 1.17 UPGMA dendrogram showing the genetic relationship of the 20 STs that belong to Leuc. lactis through MLST typing. The Phylogenetic tree was and molecular evolutionary analyses were performed using the START 2.0 software by the UPGMA method. The numbering in the figure refers to ST. Two major phylogroups were designated as A and B

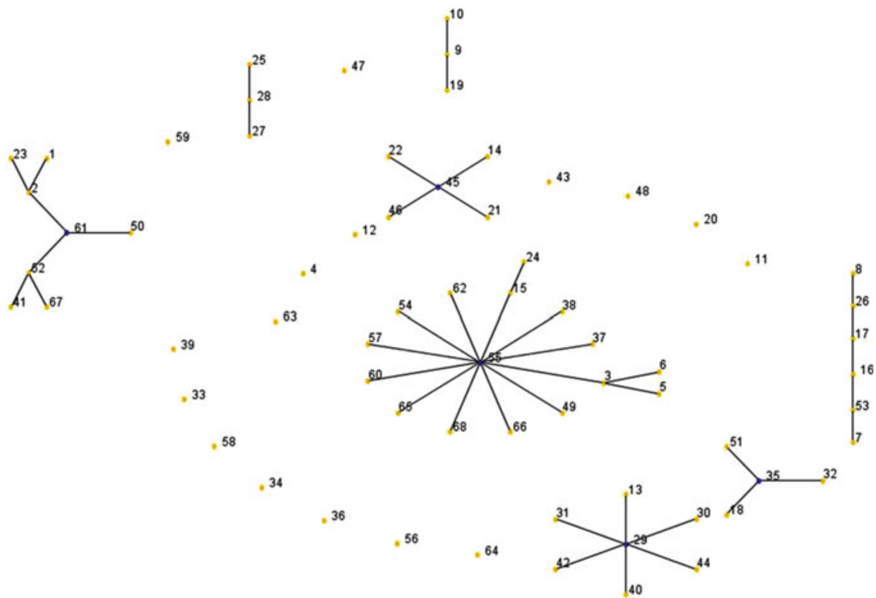


Fig. 1.18 Population snapshot of 136 *Leuc. mesenteroides* isolates. In the eBURST diagram, eight CCs and sixteen singletons are shown. ST55 is the primary founder among the eight CCs and most other isolates are SLV of ST55

indicates the linked clusters of STs with the primary founders and subgroup founders identified. Eight CCs of three or more STs were found in the eBURST diagram of the *Leuc*. *mesenteroides* isolates. ST55 CC is the largest CC. Some

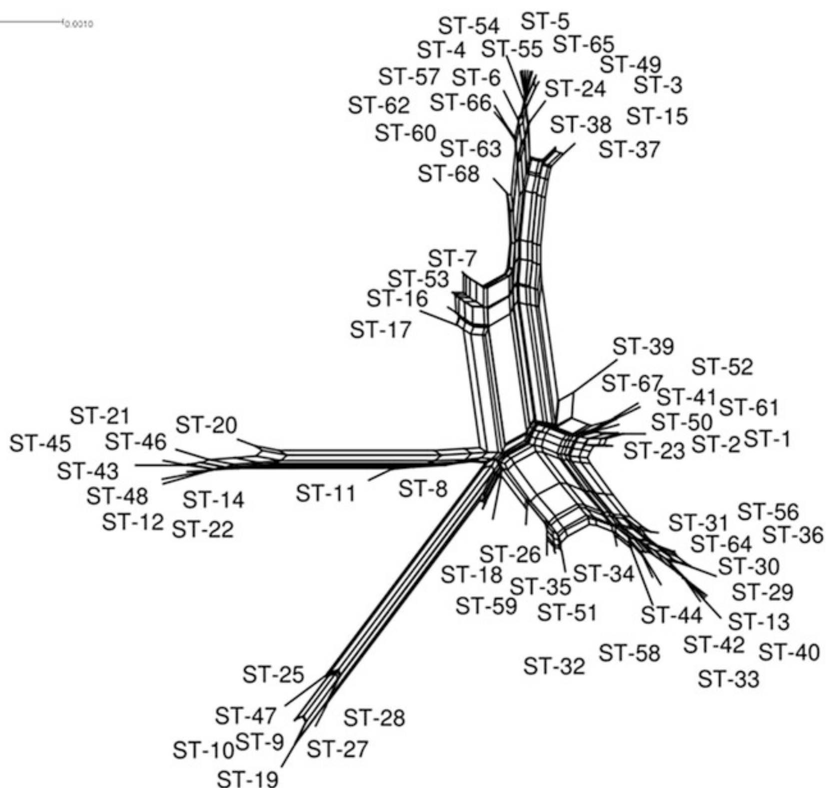


Fig. 1.19 Split decomposition analysis of 136 *Leuc. mesenteroides* isolates based on concatenated sequences of nine housekeeping genes. Formation of a parallelogram structure is suggestive of recombination

minor groups interspersed around the ST55 CC, which are typically individually unlinked STs that were not SLVs or DLVs of any other STs. The 55 CCs that are more complicated than other CCs have the 12 SLVs and 3DLVs, which have been assigned as the primary founder of the CC.

Concatenated *dna*A, *pyr*G, *rpo*B, *gro*EL, *murE*, *uvr*C, *pep*N, *mur*C and *phe*S gene sequence fragments were studied and graphically indicated using SplitsTree (Fig. 1.19). The relationship of 68 STs with the *Leuc. mesenteroides* isolates based on this analysis is described as a net-like structure with rays of different lengths. This net phylogeny is also in accordance with a recombinational population structure, and is in accordance with the observation that each strain carries its own allelic combination. 16 STs of the ST55 CC are more closely related than the other sequence types, and their tree branches are interconnected. Similar results were also found in the 8STs of the ST61 CC, 5STs of the ST45 CC, 4 STs of the ST3 CC, 7STs of the ST29 CC, etc., thereby suggesting that recombinational events between them.

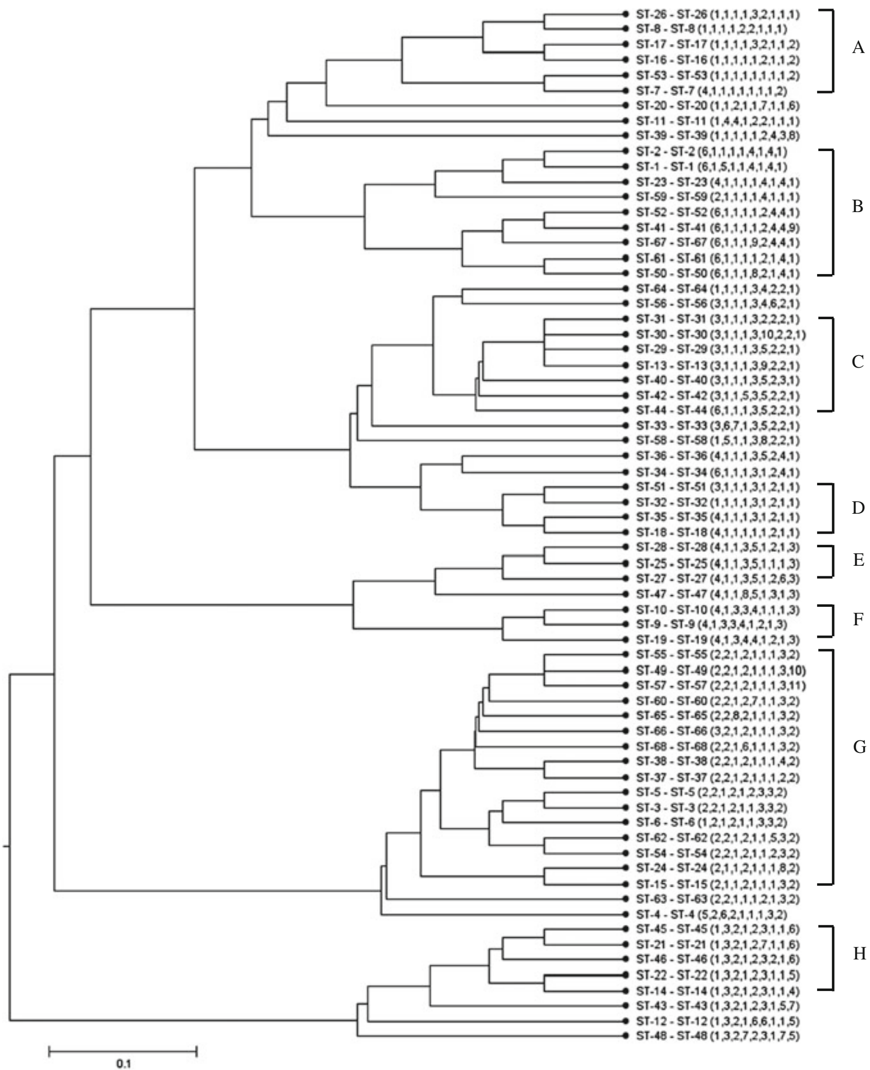


Fig. 1.20 UPGMA dendrogram showing the genetic relationship of the 68 STs that belong to 136 Leuc. mesenteroides isolates using MLST typing. Phylogenetic and molecular evolutionary analyses were performed using the START 2.0 software by UPGMA method. The numbering in the figure refers to ST. Eight major phylogroups are designated as A, B, C, D, E, F, G and H

An UPGMA dendrogram was constructed using the concatenated nucleotide sequences of the nine gene loci of the 136 *Leuc. mesenteroides* strains from various sources of Mongolia and minority area of China (Fig. 1.20). Almost all of these strains formed eight lineages, with six STs in group A, nine STs in group B, four STs in group C, four STs in group D, three STs in groups E-F, 16 STs in groups G and five STs in group H. However, 15 STs did not belong to any of the

eight groups. The abovementioned eight eBURST groups were also found in clusters in the dendrogram. Specifically, ST 59, which is as a singleton ST that was not SLVs or DLVs of any other ST, was also found in group B.

1.6.4 Conclusion

The *Leuconostoc* spp. are widespread in the natural environment and have an important function in some industrial and food fermentations. Genus *Leuconostoc* is generally considered 'generally recognized as safe' (GRAS). However, a few clinical human infections cases caused by this microorganism have been found in literature, which led to their classification as opportunistic pathogens of susceptible health-compromised individuals. In recent years, several molecular typing methods, including RAPD-PCR, TTGE, PCR-RFLP, REP-PCR, etc., have used widely to clarify *Leuconostoc* phylogeny and to identify new species. At present, 13 *Leuconostocs* spp. have been identified. MLST, as the major molecular typing method, have been used to connect the *Leuconostocs*. The *Leuconostoc* population structure in Mongolia and minority area of China appears to be quite divergent. Numerous unique sequence types among the investigated isolates were obtained. The MLST scheme developed in the study can be used for further monitoring of evolution changes and population genetics of *Leuconostoc* strains.

1.7 The Genus *Pediococcus*

1.7.1 History

The genus *Pediococcus* is typical of lactic acid bacteria (LAB) in being Gram positive, catalase negative and oxidase negative. Species in this genus grow under facultatively aerobicto microaerophilic conditions. They are homofermentative and produce lactic acid but not CO_2 from glucose and are unable to reduce nitrate (Simpson and Taguchi 1995; Holzapfel et al. 2006, 2009). They are the only LAB that divide alternately in two perpendicular directions to form tetrads (Simpson and Taguchi 1995), which thus pediococci often differ from all other LAB. They never form chains typical of the other genera of coccoid LAB such as *Leuconostoc*, *Lactococcus* and *Streptococcus* species, which also only form chains as a result of division in one plane.

The name '*Pediococcus*' was first used by Balcke in 1884 for beer-spoilage strains that had only been observed microscopically (Balcke 1884). The genus *Pediococcus* (Claussen 1903) was incorrectly cited as *Pediococcus* (Balcke 1884) in the 'Approved Lists of Bacterial Names' and also in the amended edition of this text (Euzeby 1998). Currently, the genus *Pediococcus* comprises 11 validly

Table 1.4 The list of the *Pediococcus* species

Number	Species	Isolated data	Source classification	G+C content (mol.%)
1	*P. acidilactici*	1887 (Lindner 1887)	Unknown	42.0
2	*P. argentinicus*	2008 (De Bruyne et al. 2008b)	Argentinean wheat flour	40.8
3	*P. cellicola*	2005 (Zhang et al. 2005)	Distilled-spirit-fermenting cellar	38.0
4	*P. claussenii*	2002 (Dobson et al. 2002)	Beer	40.5
5	*P. damnosus*	1903 (Claussen 1903)	Unknown	38.5
6	*P.*	*ethanolidurans*	2006 (Liu et al. 2006)	
	Argentinean wheat flour	39.5		
7	*P. inopinatus*	1988 (Back 1978)	Unknown	39.5
8	*P. parvulus*	1961 (Gunther and White 1961)	Unknown	41.0
9	*P. pentosaceus*	1934 (Mees 1934)	Unknown	38.0
10	*P. siamensis*	2007 (Tanasupawat et al. 2007)	Fermented tea leaves	42.0
11	*P. stilesii*	2006 (Franz et al. 2006)	Steeped maize grains	38.0

described species, *P. acidilactici*, *P. argentinicus*, *P. cellicola*, *P. claussenii*, *P. damnosus*, *P. ethanolidurans*, *P. inopinatus*, *P. parvulus*, *P. pentosaceus*, *P. siamensis* and *P. stilesii* (http://www.bacterio.net/pediococcus.html, 20 December 2013) (Table 1.4).

DNA G+C content (mol.%): 35–44.
Type species: *Pediococcus damnosus* (Claussen 1903).

1.7.2 Cell Morphology and Cultural Characteristics

Pediococcus species have coccoid cells that divide in two planes at right angles to each other forming tetrads, but never chains (Holzapfel et al. 2006, 2009). The cells may occur singly or in pairs, especially during the early or mid-logarithmic growth phase. The coccoid cells are perfectly spherical and rarely ovoid, ranging in diameter from 0.6 to 1.0 μm. *Pediococcus* species are nonmotile, do not form spores, are not capsulated and are characterised as Lys-D-Asp peptidoglycan types.

As typically homofermentative bacteria, *Pediococcus* species ferment carbohydrates via the Embden-Meyerhof-parnas (EMP) pathway. With the exception of *P. argentinicus* and *P. claussenii* that form L(+) lactate from glucose, the other

species all form DL(+) lactate. *Pediococcus* species are generally tolerant to high salt concentrations (growing at >18 % NaCl [w/v]) and can grow at pH values as high as 9.0 but not at pH 5.0.

1.7.3 Phylogenetic Position of the Pediococcus *Genus*

The genus *Pediococcus* is similar to *Lactobacillus* as they both belong to the family *Lactobacillaceae*, order *Lactobacillales* and phylum Firmicutes (Holzapfel et al. 2009). The phylogenetic position of the genus *Pediococcus* was established using 16S rRNA gene sequence analysis showing that the genera *Lactobacillus*, *Leuconostoc* and *Pediococcus* were closely related (Schleifer and Ludwig 1995a).

Seven genomes from *P. acidilactici*, *P. claussenii* and *P. pentosaceus* have been sequenced and published and they range is size from 1.8 to 2.0 Mb (Pittet et al. 2012; Midha et al. 2012). The complete genome of more isolates and species would help to better delineate and understand evolutionary processes in the genus.

1.7.4 Taxonomy and Phylogenesis

Characteristics generally used for species identification include the range of temperatures, pH and NaCl concentration at which growth occurs, and physiological characteristics such as how they ferment different carbohydrates, hydrolysis of arginine and which isomers of lactic acid are produced (Simpson and Taguchi 1995; Holzapfel et al. 2006, 2009). However, it is impossible to unequivocally identify *Pediococcus* species using these classical phenotypic methods. To accurately identify different molecular and biological approaches have been developed. In the last two decades, 16S rRNA sequencing has become the most reliable aid to the identification of diverse LAB (Holzapfel et al. 2001).

Based on the results of 16S rRNA sequencing, *P. dextrinicus* was found to be distantly related to *Lactobacillus casei* (Collins et al. 1991), and did not group with the other species in the genus *Pediococcus*. Subsequently, the taxonomic status of *P. dextrinicus* has been redescribed; it has now been transfered to the genus *Lactobacillus* and named *Lactobacillus dextrinicus* comb. nov. (Haakensen et al. 2009). There are two main phylogenetic branches in the genus (Fig. 1.21). Branch I comprises six species: *P. cellicola*, *P. siamensis*, *P. ethanolidurans*, *P. parvulus*, *P. damnosus* and *P. inopinatus*. Branch II comprises five species: *P. argentinicus*, *P. claussenii*, *P. stilesii*, *P. pentosaceus* and *P. acidilactici*.

Various molecular genetic methods have also been used to discriminate between species in the genus *Pediococcus* including the use of specific DNA target probes (Mora et al. 1997), amplified 16S rDNA restriction analysis (16S-ARDRA) (Rodas et al. 2003; Chenoll et al. 2003), randomly amplified polymorphic DNA (RAPD) PCR (Mora et al. 2000; Simpson et al. 2002) and pulsed-field gel

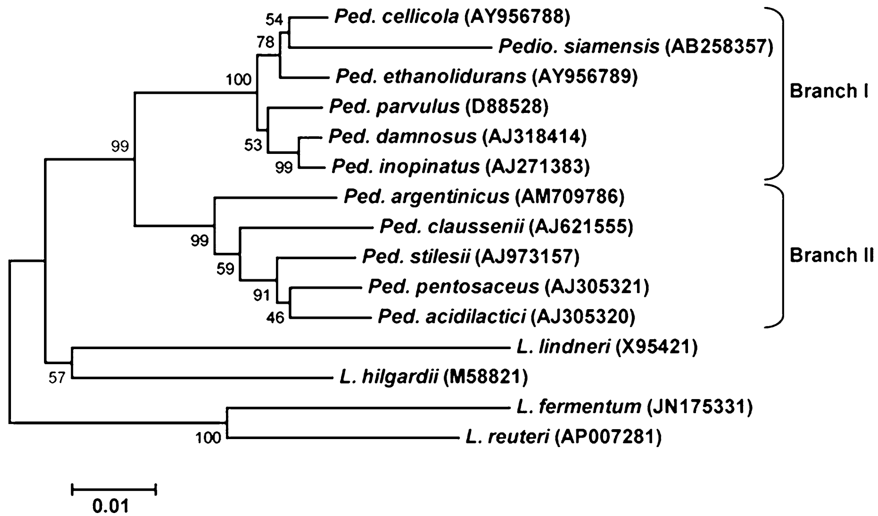


Fig. 1.21 Phylogenetic tree depicting the relationships amongst *Pediococcus* species based on 16S rRNA gene sequence. The tree was constructed through the neighbour-joining method with 1000 bootstrap replicates

electrophoresis (PFGE) (Barros et al. 2001). In 2003, Rodas et al. (2003) used the 16S-ARDRA method for identification of LAB isolated from grape must and wine. In this way, *P. parvulus* and *P. pentosaceus* were successfully identified amongst 342 LAB isolates. Another 16S-ARDRA was developed for identification of *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*.

A PCR-based RAPD technique was first used for discrimination of *P. pentosaceus* and *P. acidilactici* by Nigatu et al. (Nigatu et al. 1998) amongst 116 *Pediococcus* isolates from fermenting tefdough and fermented kocho. Mora et al. (Mora et al. 2000) also developed a RAPD molecular marker for identification of *P. acidilactici* isolates. Most other studies have used the PFGE method to discriminate among *Pediococcus* species (Barros et al. 2001). To compare RAPD PCR and PFGE techniques for species identification, Simpson et al. (2002) studied the genomic diversity of 33 strains from six species within the genus *Pediococcus* using both methods. Their results showed that both RAPD PCR and PFGE data frevealed distinct differences between the species indicating that both methods could be used for rapid classification of *Pediococcus* species isolated from a variety of sources, including food, feed, silage, beer and human clinical samples.

To accurately clarify phylogenetic relationships between more closely related *Pediococcus* species, such as *P. parvulus* and *P. damnosus*, a multi-loci sequence typing (MLST) protocol based on five housekeeping genes (*rec*A, *rpl*B, *pyr*G, *leu*S and *mle*) was developed by Calmin et al. (2008). By sequencing and comparative analysis of 19 isolates collected during wine fermentation, clear differentiation between isolates and species was possible. This result indicated that the MLST

approach could differentiate between closely related species but also provided sufficient intraspecies resolution to effectively type isolates within a species. Subsequently, to clarify the taxonomic relationships amongst *P. pentosaceus* isolates, another MLST protocol based on seven housekeeping genes, *dal*R, *gln*A, *gyr*B, *leu*S, *pgi*, *pgm* and *pyc* was developed and used to characterise a collection of 29 field-collected isolates, 1 type isolate and 1 reference isolate from food (Martino et al. 2013). In this study, 17 sequence types (ST) were identified. These results demonstrated that even if each individual gene did not identify a high number of different alleles, the combination of multiple housekeeping genes identified a high number of STs, thus providing an improved discrimination between *P. pentosaceus* isolates. Simultaneously, isolates representing nine STs were derived from vegetables, seven different STs were isolated from dairy products and a single ST was derived from a human sample demonstrating that ST types corresponded with origin, e.g. food origin or clinical origin.

1.8 The Genus *Streptococcus*

1.8.1 History

The genus *Streptococcus* consists of Gram-positive, nonmotile, spherical or ovoid cells that are typically arranged in pairs or chains when grown in liquid media. All species are facultatively anaerobic, some requiring additional CO_2 for growth. They are non-sporing, catalase negative, homofermentative and have complex and variable nutritional requirements. They metabolise carbohydrates by fermention resulting mainly in lactic acid but no gas. Their temperature optima are usually around 37 °C, but maximum and minimum temperatures vary somewhat amongst species. Many species are pathogenic to man and animals and some are highly virulent (Jones 1978; Colman et al. 1990).

In 1884, the word *Streptococcus* was first used in the generic sense to describe the type species *Streptococcus pyogenes* (Rosenbach 1884). *Streptococcus pyogenes* was originally isolated from suppurative lesions in humans; chain-forming cocci were observed in wounds and the term '*Streptococcus*' was applied to these organisms as it described their morphological arrangement (Billroth 1874). A few years later, several other *Streptococcus* species were isolated from a variety of sources including cows with mastitis, and horses and humans with pneumonia (Nocard and Mollereau 1887). The association between *Streptococcus* species and a variety of human and animal diseases was established at the turn of the last century. At this time the importance of morphologically similar bacteria, then classified as streptococci, in the dairy industry was also recognised (Schleifer and Ludwig 1995a). With progress in science and the development of molecular tools to study taxonomy and phylogeny, a large number of new species in this genus have been described and a multiplicity of names exist in the published literature.

To date, almost 100 species have been discovered in the genus *Streptococcus* (http://www.bacterio.net/s/streptococcus.html, 20 December 2013).

DNA G+C content (mol.%): 33–46.
Type species: *Streptococcus pyogenes* (Rosenbach 1884).

1.8.2 Cell Morphology and Cultural Characteristics

Streptococcus cells are normally spherical or ovoid in shape and are arranged in chains or pairs, chain formation being observed most clearly in liquid cultures. Some species, e.g. *S. mutans*, grow as short rods under some cultural conditions (Clarke 1924) and several of the other oral species appear to be pleomorphic at the time of primary isolation. Individual cells are usually 0.8–1.2 μm in diameter but chain lengths vary from a few cells to over 50 depending on the species, isolate and culture conditions.

Some species produce capsules, either of hyaluronic acid as in *S. pyogenes* (Rosenbach 1884), or of a variety of type-specific polysaccharides as in *S. pneumoniae* (Chester 1901), but this is not a consistent characteristic throughout the whole genus. Hardie and Marsh (1978) reported that several species produced extracellular polysaccharides when grown in the presence of sucrose, including both glucans and fructans.

Streptococcus species are facultatively anaerobic, and so many grow optimally under microaerophilic or anaerobic conditions; some require the addition of CO_2 to the atmosphere for growth, especially during initial isolation. Growth on solid media is often enhanced by the addition of blood, serum, or glucose. For optimal growth in liquid media, the addition of glucose or some other fermentable carbohydrate is essential, but the rapid fall in pH quickly inhibits growth unless the medium is strongly buffered, as it is in (Todd and Hewitt 1932), or the pH is controlled by the continuous addition of an alkali.

Different species produce different reactions on blood agar (Brown 1919). These different reactions were described from cultures in poured plates, but have also be observed around surface growth in stab plates and in layered blood plates (Hardie and Whiley 2006). With isolates that cause α-haemolysis, a zone of greenish discoloration occurs around the colony, usually 1–3 mm in width and the margin is indistinct. *β*-haemolysis results in a sharply defined zone of clearing around the colonies. The results of α- haemolysis resembles α-haemolysis with an obvious outer ring of clearing around the zone of discoloured (green) erythrocytes (Parker 1983). *γ*-haemolysis results in no colour change on blood agar. The different type of haemolysis are dependent upon the organisms concerned, the type of blood used (horse, sheep, human, etc.), the culture conditions and the composition of the basal medium. Haemolysis by *S. pyogenes* may be inhibited by reducing the level of sugars (Facklam and Wilkinson 1981).

1.8.3 Phylogenetic Position and Genomes

The genus *Streptococcus* is a member (type genus) of the family *Streptococaceae* and it falls within the low (<50 mol.%) G+C content *Clostridium/Bacillus* branch of Gram-positive eubacteria (Schleifer and Ludwig 1995a; Ludwig et al. 1985). Following extensive taxonomic revision of *Streptococcus* (Schlegel et al. 2003; Poyart et al. 2002; Collins et al. 1984b) and discovery of new species (Milinovich et al. 2008; Takada et al. 2010, 2013; Devriese et al. 1997; Zhang et al. 2013), the genus currently consists of over 100 species and subspecies.

The size range of completed genomes of *Streptococcus* species is about 1.8 (Bolotin et al. 2004) to 2.39 Mbp (Xu et al. 2007). Currently, more than 60 complete genomes are reported, from multiple isolates of 27 species. These species include: *S. agalactiae*, *S. anginosus*, *S. constellatus* subsp. *pharynges*, *S. dysgalactiae* subsp. *equisimilis*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, *S. gordonii*, *S. gallolyticus* subsp. *gallolyticus*, *S. infantarius* subsp. *infantarius*, *S. iniae*, *S. intermedius*, *S. lutetiensis*, *S. macedonicus*, *S. mitis*, *S. mutans*, *S. oralis*, *S. oligofermentans*, *S. parasanguinis*, *S. parauberis*, *S. pasteurianus*, *S. pneumoniae*, *S. pyogenes*, *S. salivarius*, *S. sanguinis*, *S. suis*, *S. thermophilus*, *S. uberis*.

1.8.4 Taxonomy, Phylogeny and Evolution

Streptococcus species were among the earliest bacteria to be recognised by microbiologists because of their involvement in a large number of human and animal diseases. Although many distinct taxa have been reported, their classification and nomenclature have caused considerable confusion over the years. Initially, the ability of some species of clinical importance to cause haemolysis-related changes around colonies grown on blood-containing culture media was used to distinguish species (Brown 1919; Schottmüller 1903). Haemolysis activity was useful for descriptive purposes, but differences between isolates within a species depend on incubation procedures as well as the origin of the blood in the substrate, so they were never a reliable characteristic for taxonomic subdivision. In 1906, Andrewes and Horder (1906) produced a classification for *Streptococcus* species based on morphological, serological, physiological and biochemical characteristics and it was useful for a wide range of species including human, animal, milk and environmental isolates. A few years later a keystone study on the lactic acid bacteria (LAB) isolated from dairy products was published; Orla-Jensen extended the range of tests applied to include growth under different conditions (such as varying temperature and salt concentrations), fermentation reactions and morphological characters. In 1978, Jones reviewed the composition of the genus *Streptococcus* and proposed seven groups based on artificial criteria of pathogenicity, habitat and oxygen tolerance. Phenotypic tests largely provide adequate species differentiation, but up to 13 % of isolates studied may still be identified incorrectly (Kikuchi et al. 1995). Furthermore, isolates within a given species may vary in a common

trait (Kikuchi et al. 1995; Beighton et al. 1991; Kilian et al. 1989) and the same isolate may exhibit biochemical variability under different conditions (Hillman et al. 1989; Tardif et al. 1989). Fundamentally, small differences in a phenotypic test could lead to misidentification.

Serological grouping was an important advance in the classification of *Streptococcus* species. A particular carbohydrate antigen in *S. pyogenes* was first reported by Lancefield (1933), which allowed this species to be placed within a group, Group A, of species that all had this antigen. Subsequently, identification of other antigens in other species allowed more species to be allocated to particular groups, for example, Groups B, C and G. The immunochemical properties of several of these antigens were subsequently studied in great detail. In some cases, including Group A and B, further serological subdivisions have been made based on other antigenic components, such as M, T and R proteins in Group A which have proved extremely useful for typing isolates in epidemiological investigations. Although the dectection of Lancefield group antigens has been of immense value for identification and classification of some major human and animal pathogens, this method is not species-specific (Farrow and Collins 1984a; Lawrence et al. 1985). Thus, in most cases, the mere presence of a particular antigen does not allow an isolate to be identified to species level, unless there is other supporting evidence.

To some extent, a significant contribution to *Streptococcus* taxonomy was made by Sherman who proposed a scheme for placing the isolates into four categories named 'pyogenic', 'viridans', 'lactic' and '*Enterococcus*' (Sherman 1937). The pyogenic group included the β-haemolytic species with defined group antigens (A, B, C, E, F and G). This division was not appreciably different from that of identification systems based on serogrouping. The viridans group included species that were not β-hemolytic, not tolerant to high-pH growth and salt conditions and did not grow at 10 °C. This group is still known today as the 'viridans streptococci', and many more species have been added to this group. Species in the lactic group were associated primarily with the manufacture of dairy products and not human infections. These species were reclassified as the *Lactococcus* genus (Schleifer et al. 1985). Although some isolates in the *Enterococcus* group were β-hemolytic, other characteristics such as the capacity to grow in broths at high pH, high salt concentrations and a wide temperature range (10 to 45 °C) differentiated them from the other three groups. Subsequently, the *Enterococcus* group was reclassified as the new genus *Enterococcus* (Collins et al. 1984b). As described above, only the first two of the groups currently remain in the genus *Streptococcus* as the lactic and *Enterococcus* groups having been designated as separate genera.

In the 1960s and 1970s, many scientists began to apply more modern numerical and chemotaxonomic methods to the study of the genus *Streptococcus* (Colman and Williams 1965, 1972) and more recently, molecular methods have become important taxonomic tools. These include determining the guanine–cytosine content of the DNA, DNA–DNA hybridisation analysis and sequencing of ribosomal RNA (rRNA). Based on these approaches, taxonomy of the *Streptococcus* genus has undergone dramatic changes (Schleifer 1987). A polyphasic approach

combining phenotypic and phylogenetic characterisation has clarified the situation still further, confirming the validity of many previously described species and enabling the description of others (Vandamme et al. 1996b). The anaerobic species previously included within *Streptococcus* (*S. parvulus*, *S. morbillorum*, *S. hansenii* and *S. pleomorphus*) have been excluded and assigned to other genera. *Streptococcus parvulus* was removed to the genus *Atopobium* as *Atopobium parvulum* (Collins and Wallbanks 1992), *S. hansenii* has been designated as a member of the genus *Ruminococcus* (Ezaki et al. 1994) and *S. morbillorum* was reclassified as *Gemella morbillorum* (Kilpper-Bälz and Schleifer 1988). In the case of *S. pleomorphus* (Barnes et al. 1977), 16S rRNA gene sequence catalogueing (Ludwig et al. 1988) and 16S rRNA gene sequencing (Kawamura et al. 1995) have placed this species outside the genus *Streptococcus* and demonstrated that it is most closely related to *Clostridium inocuum*. Recent analysis has placed *S. pleomorphus* within the family *Erysipelotrichaeae* together with *Eubacterium biforme* and *Eubacterium cylindroids* (Holzapfel et al. 1872).

Different approaches have been used to infer phylogenetic relationships amongst species from the genus *Streptococcus*. In particular, phylogenetic analysis based on comparative 16S rRNA analysis has helped to clarify intrageneric relationships of the genus and 16S rRNA gene sequencing is still a rapid tool for gaining insight into the taxonomic position of an unknown isolate. By 16S rRNA gene sequence analysis and reassociation data the species within the genus *Streptococcus* sensu stricto comprise some distinct ‘species groups’ and also several species that remain independent and ungrouped. The species groups have been designated as ‘pyogenic’, ‘bovis’, ‘mutans’, ‘mitis’, ‘anginosus’ and ‘salivarius’. Figure 1.22 shows a phylogenetic tree of the currently available sequences for the species included in the *Streptococcus* genus.

The species within the present pyogenic group include *S. agalactiae*, *S. canis*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, *S. hyointestinalis*, *S. iniae*, *S. parauberis*, *S. pluranimalium*, *S. pyogenes*, *S. uberis*, *S. urinalis*, *S. phocae*, *S. didelphis* with strains from Lancefield groups C, G and L and groups E, P, U and V including *S. dysgalactiae* subsp. *dysgalactiae*, *S. dysgalactiae* subsp. *equisimilis* and *S. porcinus*, respectively. *Streptococcus parauberis* and *S. uberis* strains were previously both included as distinct genotypes within *S. uberis* by DNA–DNA hybridisation and designated *S. uberis* types I and II, respectively (Collins et al. 1984a; Fuller et al. 2001). 16S rRNA gene sequence analysis later demonstrated that type I and II isolates were sufficiently different to warrant separate species status (Williams and Collins 1990). *Streptococcus dysgalactiae* and *S. equisimilis* were designed as belonged within a single species (Farrow and Collins 1984a; Kilpper-Bälz and Schleifer 1984) for which the name *S. dysgalactiae* was proposed and an amended description given. In 1996, Vandamme et al. showed that *S. dysgalactiae* and *S. equisimilis* could be allocated into subspecies based on their physiological and biochemical properties and by numerical analysis of whole-cell protein profiles. Finally, *S. dysgalactiae* subsp. *equisimilis* was proposed for human-derived isolates belonging to groups C and G, and isolates of animal origin were placed in a separate subspecies for which the name *S. dysgalactiae* subsp.

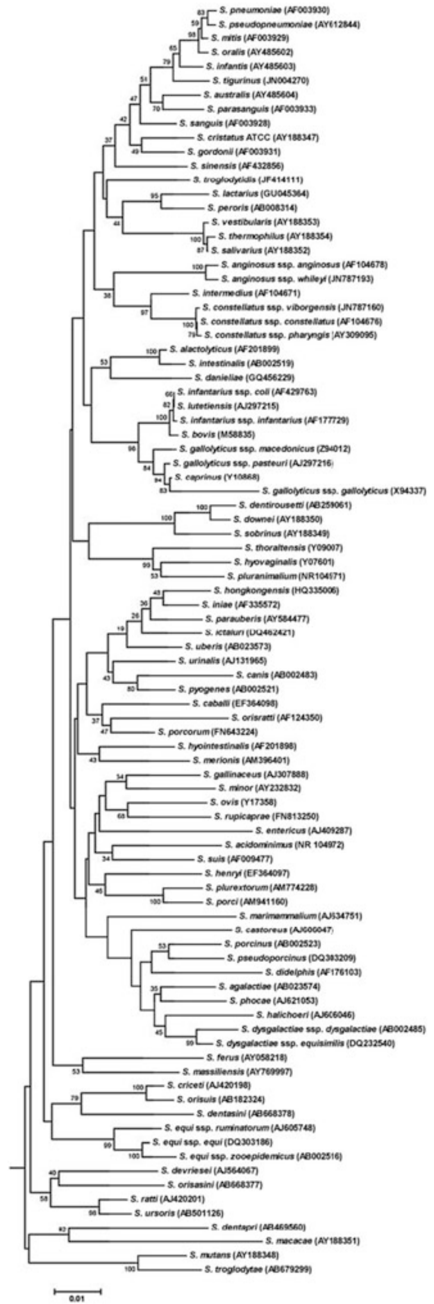


Fig. 1.22 Phylogenetic relationships between species in the genus *Streptococcu* based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method

dysgalactiae was proposed (Vandamme et al. 1996a). In 1998, Vierira et al. used phenotypic characterisation, DNA–DNA reassociation and multilocus enzyme electrophoresis (MEE) to describe these two subspecies within *S. dysgalactiae* which was in close agreement with Vandamme et al. In addition, *S. equi* and *S. zooepidemicus* are now regarded as two subspecies of *S. equi* based on their phenotypic and genotypic characterisation (Farrow and Collins 1984a; Poyart et al. 1998).

The mutans groups are associated with dental plaque in humans and several other animal species. 16S rRNA data place *S. criceti*, *S. downei*, *S. macacae*, *S. mutans*, *S. ratti*, *S. sobrinus*, *S. ferus*, *S. orisratti* and *S. devriesei* together within this group (Kawamura et al. 1995; Bentley et al. 1991; Zhu et al. 2000; Collins et al. 2004).

The anginous group includes *S. anginosus* subsp. *anginosus*, *S. anginosus* subsp. *whileyi*, *S. constellatus* subsp. *constellatus*, *S. constellatus* subsp. *pharynges*, *S. constellatus* subsp. *viborgensis* and *S. intermedius*, which are isolated from the oral cavity, upper respiratory, intestinal and urogenital tracts. According to Coykendall et al. (1987), *S. constellatus* is a later heterotypic synonym of *S. anginosus*. In 1991, Whiley and Beighton (Whiley and Beighton 1991) amended the descriptions and recognised *S. constellatus* and *S. anginosus* as distinct species. Subsequently, it has been proposed that *S. constellatus* is comprised of two subspecies, *S. constellatus* subsp. *constellatus*, isolated from a relatively broad clinical background and *S. constellatus* subsp. *pharynges*, mainly from the human throat and from individuals with pharyngitis (Whiley et al. 1999). The other subspecies *S. constellatus* subsp. *viborgensis* was also isolated from patients with sore throats (Jensen et al. 2012). In 2013, *S. anginosus* was divided into two subspecies, *S. anginosus* subsp. *anginosus* and *S. anginosus* subsp. *whileyi* using multilocus sequence analysis (MLSA) combined with 16S rRNA gene sequence and phenotypic analyses (Jensen et al. 2012).

Streptococcus salivarius, *S. thermophilus* and *S. vestibularis* are members of the salivarius group, however, the taxonomy of *S. thermophilus* has been controversial. Initially, Farrow and Collins made a proposal for recognition of *S. salivarius* and *S. thermophilus* as subsp. *salivarius* and subsp. *thermophilus*, respectively (Farrow and Collins 1984b). However, further DNA hybridisation studies showed that it was clustered with the oral species *S. salivarius* and *S. vestibularis* but that assignment of separate species status for *S. thermophilus* was justified (Schleifer et al. 1919).

The mitis group currently includes *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. mitis*, *S. oralis*, *S. parasanguinis*, *S. peroris*, *S. pneumoniae*, *S. sinensis* and *S. sanguini.* These strains are mainly isolated from the healthy oral and pharyngeal tracts in humans.

The species within the bovis group include *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *macedonicus*, *S. gallolyticus* subsp. *pasteurianus*, *S. bovis*, *S. infantarius* subsp. *coli*, *S. infantarius* subsp. *infantarius* and *S. alactolyticus*. In 1984, Farrow et al. found that *S. bovis* and *S. equines* were genetically very closely related (>70 % DNA homology) and represented a single species (Farrow et al. 1984).

Later studies confirmed this result using biochemical traits, DNA–DNA hybridisation experiments and examination of a noticeable divergence in 16S rDNA sequences and the manganese-dependent superoxide dismutase gene (*sod*A) (Poyart et al. 2002). A recent study using DNA–DNA reassociation and gene sequence data support the inclusion of *S. macedonicus* and *S. waius* within *S. gallolyticus* (Poyart et al. 2002), even though these two species of dairy origin were originally characterised by 16S-23S intergenic spacer sequence analysis, random amplified polymorphic DNA fingerprinting, PFGE analysis and DNA–DNA reassociation experiments. All genetic data suggests that *S. waius* belongs to the species described previously as *S. macedonicus* (Manachini et al. 2002). Further, Schlege et al. have proposed that isolates previously classified as *S. pasteurianus* should be included within *S. gallolyticus* with the proposed recognition of *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *macedonicus*, *S. gallolyticus* subsp. *pasteurianus*. The other species not assigned to recognised species groups may have separate lines of descent, or are only weakly affiliated to one of the main species groups listed above.

DNA fingerprinting technologies have also been successfully applied to molecular-typing and for discriminating between closely related *Streptococcus* species. These technologies include restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE). Jayarao et al. successfully identified and differentiated between *Streptococcus* and *Enterococcus* species by RFLP analysis of the 16S rRNA gene (Jayarao et al. 1992). Barsott et al. demonstrated the usefulness of PCR–RFLP analysis of the 16S–23S intergenic spacer for identifying and differentiating amongst *S. mitis* group species (Barsotti et al. 2002). Several scientists have used RAPD profiles to characterise and type *Streptococcus* isolates (Seppälä et al. 1994; Martinez et al. 2000; Chatellier et al. 1997; Truong et al. 2000). Lazzi et al. successfully used AFLP to analyse the genetic diversity amongst *S. thermophilus* isolates from different sources (Lazzi et al. 2009). Similarly, Jacobs et al. used AFLP to reveal discriminatory and reproducible patterns in *S. anginosus* group isolates (Jacobs et al. 2003). PFGE is an effective tool for typing *S. pneumoniae* isolates that is capable of subdividing serotypes and tracing the origins of individual isolates (Lefevre et al. 1993). These methods have good discriminatory power and can be scaled up to characterise a large number of bacterial isolates at low cost (Boers et al. 2012), but they only describe genetic differences based on fingerprints and are less useful for precisely defining the underlying phylogenetic relationships (Cai et al. 2007).

Recently, more protein encoding genes have been identified and used for classification of closely related species and for determining the phylogenetic position of new species. Sequence analysis of internal fragments of the manganese-dependent superoxide dismutase gene (*sod*A_{int}) has considerable potential as a gene-based approach to identification (Poyart et al. 1998; Kawamura et al. 1999; Whatmore and Whiley 2002; Hoshino et al. 2005). A comparison of sequence similarities between the 16S rRNA gene and *sod*A_{int} has shown the latter to be an

alternate DNA target for species-specific identification, particularly for differentiating between closely related species such as *S. mitis*, *S. oralis* and *S. pneumoniae* within the mitis group. Täpp et al. analysed the gene encoding the RNA subunit of endoribonuclease P (*rnp*B), and found it to be potentially extremely useful for phylogenetic analysis and species discrimination (Täpp et al. 2003). A similar tree topology to that derived from 16S rRNA was obtains for *rnp*B and it was concluded that a combination of 16S rRNA gene and *rnp*B gene sequence analysis would result in a better phylogenetic understanding of the genus *Streptococcus*. Moreover, the *dna*J gene (a member of the Hsp 70 family) and the *gry*B gene (encoding the B-subunit protein of DNA gyrase) also show sufficient intraspecies divergence to be a valuable tool for identification and discriminating between closely related isolates within a species (Itoh et al. 2006; Maeda et al. 2011). However, studies indicate that reliance on data from the sequence of single genes for discrimination may result in a degree of misidentification for some isolatess due to previous horizontal gene transfer events, notably within the species *S. sanguinis*, from the anginosus group. Therefore, some authors now recommend multilocus sequence typing (MLST) to discriminate between closely related species and analyse genetic diversity and phylogenetic relationships.

Multilocus sequence typing (MLST), in which conserved housekeeping genes are sequenced, provides fast and relatively cheap nucleotide sequence determination. Because typing is based on nucleotide sequence information, data can be shared amongst databases around the world and provide accurate information on isolate evolution (Enright and Spratt 1999). Therefore, MLST has became the gold standard for studying the evolution and population genetics of pathogenic microbes (Maiden 2006). It has been used successfully to identify and type many pathogenic *Streptococcus* species, such as *S. suis* (King et al. 2002), *S. pneumoniae* (Enright and Spratt 1998; Feil et al. 2000), *S. pyogenes* (Enright et al. 2001) and Group B *Streptococcus* (Jones et al. 2003). These studies have confirmed that MLST is a powerful method to characterise *Streptococcus* species and probe aspects of the population and evolutionary biology of these organisms.

Streptococcus thermophilus is a ‘generally recognised as safe’ (GRAS) species that is essential for the manufacture of many types of fermented dairy products. However, information on the genetic diversity and population structure of this food bacterium was scarce. In 2010, genetic diversity within the salivarius group was studied using MLST (Delorme et al. 2010). Twenty six *S. thermophilus* isolates from different products (cheese, yogurt, fermented milk and starter cultures) collected in 11 countries over a 40-year period (1962 to 2002), and one isolated from human blood, were included in these analyses. Seven housekeeping genes (*ilv*C, *pep*O, *pyr*E, *glc*K, *ddl*A, *thr*S, *dna*E) were amplified and sequenced. The analysis of the allelic profiles of the *S. thermophilus* isolates revealed no significant clustering suggesting no correlations between the sequence type, geographic origin and the type of products from which the isolates originated.

In 2013, a different MLST scheme was developed for *S. thermophilus* and used to investigate the genetic polymorphisms and evolutionary relationships amongst isolates by a research team in the Key Laboratory of Dairy Biotechnology and

Engineering, Ministry of Education, P. R. China (Yu et al. unpublished data). A total of 239 isolates of *S. thermophilus* from different home-made fermented dairy foods (cow's milk, yak's milk, goat's milk, mare's milk and a type of traditional cheese called Qula) in six provinces of China and 11 provinces and 1 city of Mongolia between 2005 and 2009. Ten housekeeping genes, *car*B, *clp*X, *dna*A, *mur*C, *mur*E, *pep*N, *pep*X, *pyr*G, *rec*A and *rpo*B, ranging in size from 451 to 629 bp, were used for MLST analysis. Different allelic sequences (with at least one nucleotide difference) were assigned arbitrary numbers. For each of the ten MLST loci, a unique nucleotide sequence defined an allele. Unique allelic profiles, consisting of the allele numbers at each of the ten MLST loci, defined sequence types (STs). In total, 107 different STs (ST1–ST107) were obtained; ST5 was comprised of the largest number of isolates, 15 (6.3 % of all isolates) followed by ST2 and ST79 (13 isolates [5.4 %] each) and ST39 (11 isolates, 4.6 %).

Split-decomposition analysis was used to detect the conflict that recombination introduces among nucleotide sites in sequence data (Huson 1998). The split graphs of each locus showed starlike or slight network structures, indicating that most of the genes were affected by intragenic recombination (Fig. 1.23a). The concatenated sequence of the 10 MLST genes also showed a network-like structure (Fig. 1.23b), suggesting that most sections among the 10 genes had widespread associative recombination. The d_N/d_S ratio of 10 loci ranged from 0.0440 (*pyrG*) to 0.1761 (*pepN*) and all were less than 1, suggesting strong purifying selective pressure (negative selection) in these genes. Intergenic recombination was quantified by estimating the linkage disequilibrium amongst the 10 loci using I_A and I_A^S (Haubold and Hudson 2000). The I_A^S value in this study (0.0915) confirming that recombination played a key role in the evolution of the analyzed genes. In summary, recombination and selective pressure were likely to have contributed to the evolution of *S. thermophilus*, which was in agreement with previous reports on recombination and horizontal gene transfer (HGT) events contributing to the plasticity of the *S. thermophilus* genome (Bolotin et al. 2004; Lefébure and Stanhope 2007).

To explore relationships amongst 239 *S. thermophilus* isolates at the micro-evolutionary level, Assignment of STs to clonal complex (CC) was done by eBURST analysis. 107 STs divided into 16 CCs (CC1–CC16) and 31 singletons (Fig. 1.24). The eBURST analyses showed that the evolution of *S. thermophilus* isolates was correlated with geographic environment, such as climate and altitude. The reason for this is likely to be because isolates from the same region are likely to have been exposed to similar environmental selective pressures. Furthermore, no significant associations between clusters and the type of dairy products were found in our collection of *S. thermophilus*, which was probably due to an unequal number of isolates from different fermented products.

In recent years, the number of genome sequences available for *Streptococcus* species is increasing and revealing more important insights. Comparison of genome sequences from bacteria is expected to provide an indication of their evolutionary dynamics and allow a really 'natural' classification scheme to be developed (Felis and Dellaglio 2007). In 2002, Tettelin et al. sequenced the

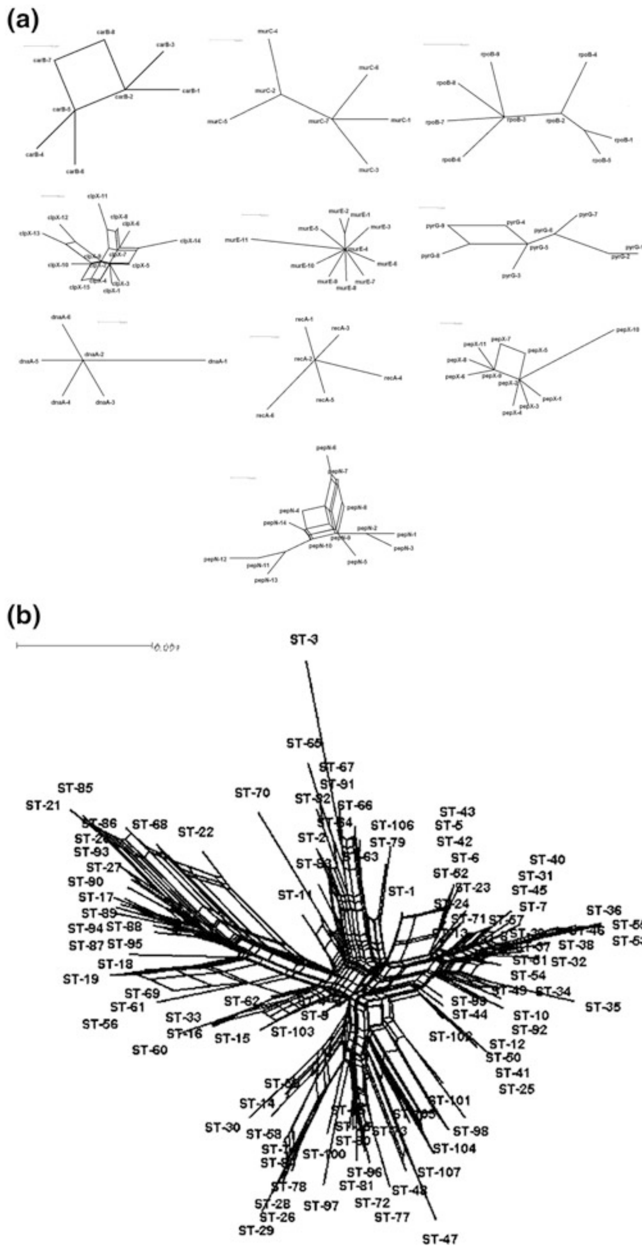


Fig. 1.23 Split decomposition analysis based on allelic profiles of *Streptococcus thermophilus* isolates. **a** Split decomposition of alleles for individual MLST loci. **b** Combined split decomposition of alleles for all ten MLST loci

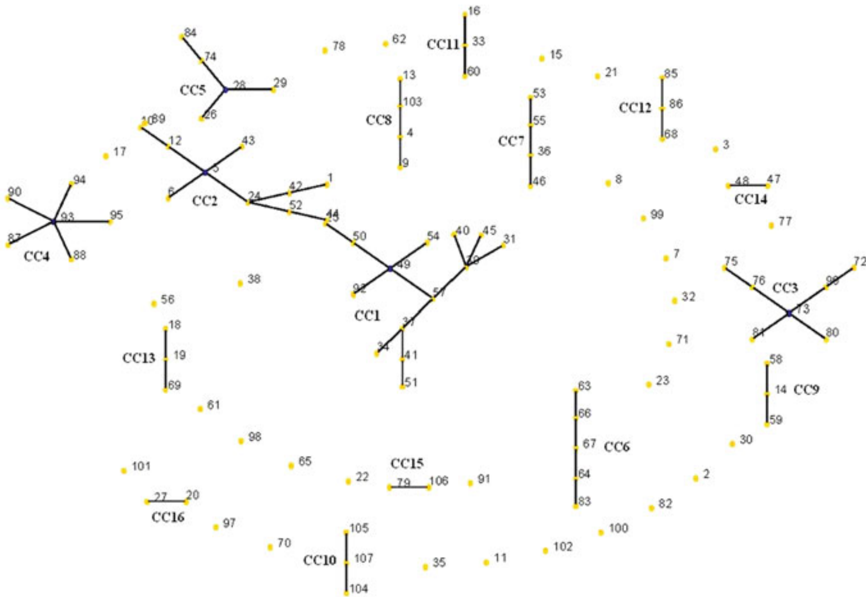


Fig. 1.24 eBURST analysis map of 239 *S. thermophilus* isolates based on allelic profiles of ten genes *car*B, *clp*X, *dna*A, *mur*C, *mur*E, *pep*N, *pep*X, *pyr*G, *rec*A and *rpo*B

complete genome of the human pathogen, serotype V *S. agalactiae* and comparative genomic analysis of this species revealed genetic heterogeneity amongst *S. agalactiae* isolates, even when they were from the same serotype, and provided insights into evolution of the virulence mechanism (Tettelin et al. 2002).

In 2004, Bolotin et al. completely sequenced and then compared the genomes of two isolates of the dairy bacterium, *S. thermophilus* (isolates LMG18311 and CNRZ1061), revealing a striking level of gene decay (10 % pseudogenes) in both isolates. They concluded that *S. thermophilus* has evolved, mainly, through loss-of-function events that remarkably mirror the environment of their dairy niche, and result in severe reductions in virulence (Felis and Dellaglio 2007). Although comparative whole genomic analysis could highlight differences in gene content and order on the chromosome, provide a phylogenetic analysis (based on virtually all available genes) and determine nucleotide composition at different taxonomic levels, it is not useful in analysing large numbers of isolates due to of the expense of equipment and manpower.

1.8.5 Conclusion

The genus *Streptococcus* comprises a wide variety of pathogenic and commensal Gram-positive bacteria. They inhabit a wide range of hosts including humans, horses, pigs and cows. For this reason, it has been essential to elucidate the

phylogeny and evolution of *Streptococcus* species to underpin epidemiology studies. Nowadays, a number of molecular typing methods have been used to reveal the phylogeny and evolution of the genus *Streptococcus*. However, no single molecular approach is perfect and we hope to develop better methods to provide a systematic and comprehensive understanding of phylogenic relationships and evolutionary history of species in the *Streptococcus* genus.

1.9 The Genus *Weissella*

1.9.1 History

In 1993, the genus *Weissella* was first proposed on the basis of taxonomical studies on some unknown *Leuconostoc*-like organisms from fermented Greek sausage (Collins et al. 1993). At that time, the established new genus encompassed the *Paramesenteroides* group including formerly classfied *Leuconostoc paramesenteroides*, *Lactobacillus kandleri*, *L viridescens*, *L. halotolerans*, *L. confusus* and *Weissella hellenica* (Collins et al. 1993). Those included species were assigned to either *Lactobacillus* or *Leuconostoc*, and were presented as a distinct phylogenetic cluster of heterofermentative lactic acid bacteria (LAB). Nowadays, morphologically different representatives were included and the members increased to eighteen species (http://www.bacterio.net/weissella.html, 20 December 2013), which are *W. thailandensis*, *W. cibaria*, *W. hellenica*, *W. minor*, *W. viridescens*, *W. paramesenteroides*, *W. confusa*, *W. soli*, *W. koreensis*, *W. kandleri*, *W. ghanensis*, *W. beninensis*, *W. fabaria W. halotolerans*, *W. oryzae*, *W. diestrammenae*, *W. ceti* and *W. fabalis* (Bjorkroth et al. 2002; Choi et al. 2002; De Bruyne et al. 2009; De Bruyne et al. 2008; Ennahar and Cai 2004; Lee et al. 2002; Magnusson et al. 2002; Padonou et al. 2009; Tanasupawat et al. 2000; Oh et al. 2013; Snauwaert et al. 2012; Tohno et al. 2012; Vela et al. 2011).

As documented in the publications, *Weissella* strains were widely detected in a variety of sources such as kimchi, fermented fish products, sourdough, human faeces, field grass and intestinal contents of ducks (Di Cagno et al. 2006; Walter et al. 2001; Samelis et al. 1998; Kurzak et al. 1998). Not surprisingly, a bacterium isolated from garden soil was showed to represent a new species of *Weissella* (Magnusson et al. 2002). However, it seems unclear where it was originated. Recently, there are growing interests on its potential health-promoting effects. Various screening tests performed on this genus showed that they were able to adhere to epithelial cells (Kang et al. 2005), produce glucan, fructan and bacteriocin (Di Cagno et al. 2006), exhibit antimicrobial activity (Lee 2005), inhibit the in vitro formation of *Streptococcus mutans* biofilms as well as the in vivo formation of oral biofilms (Kang et al. 2006). Some of them were used as specific probiotics for flounders due to their excellent exhibition of antimicrobial activity against fish pathogens such as *Edwardsiella*, *Pasteurella*, *Aeromonas* and *Vibrio* (Cai et al. 1998).

1.9.2 Cell Morphology and Biochemical Characteristics

The overall description of the genus *Weissella* include strains that are Gram-positive, non-spore forming, heterofermentative and usually non-motile. The only motile species with peritrichous flagella, *W. beninensis*, was isolated from submerged cassava fermentations, as described by Padonou et al. (Padonou et al. 2009) Typical strains from this genus can be classified as two different morphological types: the asporogenous short rods with rounded tapered end or ovoid, occurring singly, in pairs or in short chains. For the first time, a LAB genus has morphology of both rods and cocci, suggesting that morphology is a poor indicator for classification.

As heterofermentative bacteria, *Weissella* ferment carbohydrates via the hexosemonophosphate and phosphoketolase pathways. The end products of glucose fermentation contain CO_2, ethanol and acetate. *Weissella* grows well at 15 °C; optimal growth of some species occurs at 42–45 °C. In the presence of 4, 6.5 and 10 % of NaCl, growth of *W. beninensis*, *W. cibaria* and *W. thailandensis* strains can be observed (Bjorkroth et al. 2002; Padonou et al. 2009; Tanasupawat et al. 2000). The phenotypic characteristics, sugar fermentation patterns, NH_3 from arginine, dextran formation, lactic acid configuration and DNA G+C content were previously reported and summarised in Table 1.5 (Collins et al. 1993; Bjorkroth et al. 2002; Choi et al. 2002; De Bruyne et al. 2009; De Bruyne et al. 2004, 2008a; Lee et al. 2002; Magnusson et al. 2002; Padonou et al. 2009; Ennahar and Cai Tanasupawat et al. 2000; Oh et al. 2013; Snauwaert et al. 2012; Tohno et al. 2012; Vela et al. 2011; Figueiredo et al. 2011). Most of the listed species can produce acid from maltose, ribose and sucrose, and it is interesting to note the wide substrate carbohydrate diversity of *W. soli* and *W. beninensis*, which can produce acid from most of the listed carbohydrates. Hydrolysis of aesculin was seen in *W. minor*, *W. confusa* and *W. paramesenteroides*. *W. kandler*, *W. confusa* and *W. koreensis* sp.nov can produce dextran from the growth media. With the exception of *W. paramesenteroides, W. hellenica, W. thailandensis, W. koreensis, W. soli*, *W. ghanensis*, *W. oryzae*, *W. diestrammenae*, *W. ceti* and *W. fabalis*, *W. kandler, W. viridescens, W. minor, W. halotolerans, W. confusa, W. cibaria, W. beninensis and W. fabaria* species of the genus produce DL lactate from glucose. The cell wall murein is mainly composed of lysine with an interpeptide bridge containing alanine, or serine and alanine (Bjorkroth et al. 2002; Choi et al. 2002; De Bruyne et al. 2009; De Bruyne et al. 2008a; Ennahar and Cai 2004; Lee et al. 2002; Magnusson et al. 2002; Padonou et al. 2009; Tanasupawat et al. 2000; Oh et al. 2013; Snauwaert et al. 2012; Tohno et al. 2012; Vela et al. 2011). The DNA G+C content ranges from 35 to 47 %. Production of dextran has been recorded for *W. kandler*, *W. confusa*, *W. koreensis*, *W. ghanensis, W. beninensis*, *W. fabaria*, *W. oryzae* and *W. fabalis*.

Table 1.5 Differentiation of *Weissella* from other lactic acid bacteria

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Acid produced from																		
L-Arabinose	−	−	−	−	−	d	+	+	+	+	+	+	−	-	+	+	−	−
Cellobiose	−	−	+	−	+	d	−	−	−	+	−	+	+	+	−	NT	−	+
Galactose	+	−	−	−	+	+	−	−	−	−	−	−	+	−	+	−	−	−
Maltose	−	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+	+	+
Melibiose	−	−	−	−	−	+	−	+	−	−	+	−	+	−	−	NT	−	−
Raffinose	−	−	−	−	−	d	−	+	−	−	+	−	+	−	−	NT	−	−
Ribose	+	−	+	+	+	d	−	+	+	−	+	−	+	−	+	+	+	−
Sucrose	−	d	+	−	+	+	+	+	−	−	+	+	+	−	−	NT	−	−
Trehalose	−	d	+	−	−	+	+	+	−	−	+	+	+	+	+	NT	+	+
Xylose	−	−	−	−	+	d	−	−	+	+	+	−	−	−	−	+	−	−
Hydrolysis of aesculin	−	−	+	−	+	+	NT	+	−	+	+	+	+	+	−	+	−	+
NH_3 from arginine	+	−	+	+	+	−	−	−	−	+	+		+	+	−	+	+	+
Dextran formation	+	NT	−	NT	+	−	−	−	+	+	−	+	+	+	+	NT	−	+
Lactic acid configuration	DL	DL	DL	DL	DL	D	D	D	D	DL	D	D	DL	DL	D	D	D	D
DNA G+C content (mol.%)	39	41–44	44	45	45–47	37–38	39–40	38–41	37	44–45	43	40	37	38	40.6	45	39.2	37

* Numbers 1–14 stand for the fourteen species *W. kandler*, *W. viridescens*, *W. minor*, *W.halotolerans*, *W. confusa*, *W. paramesenteroides*, *W. hellenica*, *W. thailandensis*, *W. koreensis*, *W. cibaria*, *W. soli*, *W. ghanensis*, *W. beninensis*, *W. fabaria*, *W. oryzae*, *W. diestrammenae*, *W. ceti* and *W. fabalis*. Scored as D, >90 % of the lactic acid is D(−); DL, >25 % of the total lactic acid is L(+). d 11–89 % of the strains positive. NT not tested

1.9.3 Phylogenetic Position and Genomes

The phylogenetic position of *Weissella* within the phylum *Firmicutes* was established by 16S rRNA gene sequences analysis, which is placed within the family of *Leuconostocaceae*. The *Weissella* genus has been transferred from some of the members of *Leuconostoc* and *Lactobacillus*. It can be easily imagined that the relationship between certain species from *Lactobacllus*, *Leuconostoc* and *Weissella* are close. As a matter of fact, *Oenococcus*, *Leuconostoc* and *Weissella* are in the same clade.

The size of *Weissella* genome ranges from 1.35 to 2.32 Mbp, with 0 to 3 plasmids in the cell (Amari et al. 2012; Benomar et al. 2011; Kim et al. 2010; Ladner et al. 2013; Lee et al. 2012a, 2011). At the time of writing, 9 genomes from 6 different species were published. These species include *W. confusa*, *W. fabaria*, *W. thailandensis*, *W. cibaria*, *W. ceti* and *W. koreensis*. Most of the strains included are isolated from food source, except for *W. ceti* NC36, which is an emerging pathogen of farmed rainbow trout in the United States (Ladner et al. 2013).

1.9.4 Taxonomy and Phylogenesis

As expected, it is impossible to unequivocally identify *Weissella* species by classical phenotypic methods. For reliable identification of these species, different molecular and biological approaches were developed one after another. Such approaches include whole-cell protein analysis, restriction fragment length polymorphism analysis, fluorescent amplified fragment length polymorphism fingerprinting of whole genomes, denaturing gradient gel electrophoresis and random amplified polymorphic DNA-PCR (Bjorkroth et al. 2002; De Bruyne et al. 2009; Kurzak et al. 1998; Ampe et al. 1999; Jang et al. 2002).

Based on the results of a 16S rRNA analysis, phylogeny of the genus *Weissella* was first established by Collins et al. (1993) This genus forms a phylogenetically coherent group among the lactic acid bacteria, which separated from the genus *Leuconostoc* (Martinez-Murcia and Collins 1991; Martinez-Murcia and Collins 1990). As seen from Fig. 1.25, four main phylogenetic branches exist in the 16S rRNA gene-based tree. *W. koreensis* is closer to *W. kandleri*, whilst *W. viridescents* is closer to *W. minor*. Close relationship could also be observed between *W. fabaria* and *W. ghanensis*, as do *W. confusa* and *W. cibaria*. The remaining species like *W. hellenica*, *W. paramesenteroides* and *W. thailandensis* are positioned in a separate branch, but share the same or similar properties at the biochemical level. To differentiate these bacterial species, Schillinger et al. (2008) developed a genus-specific PCR analysis method. They designed primer sets targeted specifically at regions of the 16S rRNA genes and the specificity of the PCR was evaluated using the type strains of *Weissella*. The accuracy of this *Weissella*-specific primer based PCR approach was confirmed by both repetitive extragenic

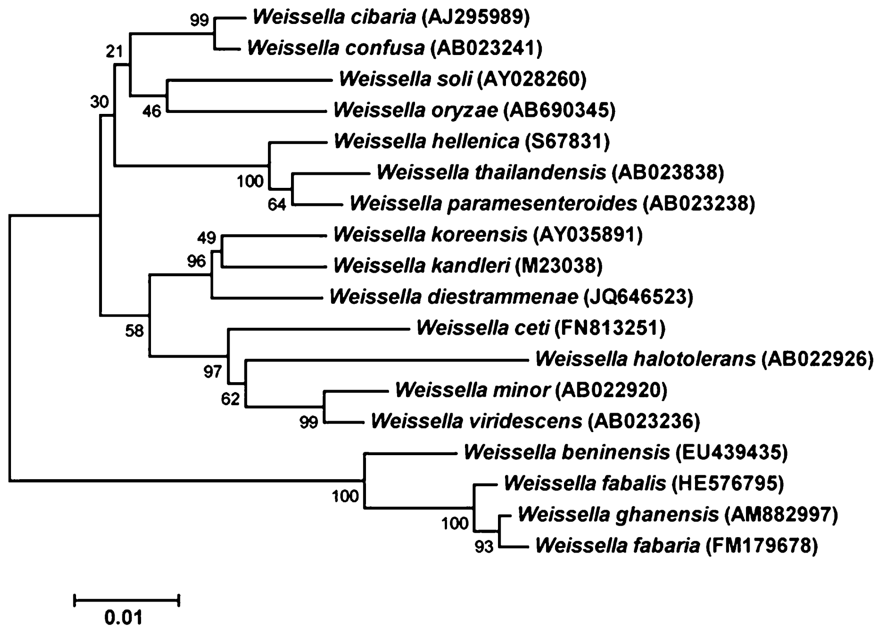


Fig. 1.25 Phylogenetic tree based on the 16S rRNA of *Weissella* type strains. The tree was constructed through the neighbour-joining method

palindromic-PCR and 16S rRNA gene sequencing (Schillinger et al. 2008). For some other *Weissella* species, species-specific sequences are located in helix 1007/1022 of the variable region V6 in the 16S rRNA gene (Collins et al. 1993). A method based on the generation of a 725 bp 16S rDNA fragment after restriction enzyme digestion was developed (Jang et al. 2002).

Besides 16S rRNA gene, phylogenetic analyses of multiple housekeeping genes or macromolecules were also conducted on *Weissella* strains. De Bruyne et al. (2009) reported the unique taxonomic position of *W. fabari*. Additional sequence analysis of *phe*S gene proved its useful role for identification of all *Weissella-Leuconostoc-Oenococcus* species and novel species (De Bruyne et al. 2009). The large discriminatory power of *phe*S gene compared with 16S rRNA gene sequences was confirmed by analyses of *phe*S genes from 29 *Weissella* and 6 *Oenococcus* strains (De Bruyne et al. 2009). Previously, De Bruyne et al. (2007) have revealed that, except for the subspecies of *Leuconostoc mesenteroides*, the interspecies variation for the *phe*S gene is much greater than that of the 16S rRNA gene. In a similar study, Arahal et al. (2008) indicated that *rec*N used either alone or in combination with 16 s rRNA data can serve as a phylogentic marker as well as a tool for species identification. In order to verify the discriminatory power of *rec*N, partial sequences from 23 type strains and several additional strains were amplified following subsequent sequencing. Phylogeny was evaluated based on neighbour joining, maximum likelihood and maximum parsimony methods with

the inclusion of variability filters (Arahal et al. 2008). For evaluating the phylogenetic structure of the *Leuconostoc*-*Oenococcus*-*Weissella* clade, comparative analysis of 16S rRNA, *dna*A, *gyr*B, *rpo*C and *dna*K genes was performed by Chelo et al. (2007). It is noteworthy that phylogenies obtained with the different genes were in overall good agreement. Moreover, a well-supported and almost fully resolved phylogenetic tree was obtained when the combined data were analysed in a Bayesian approach. Furthermore, the evolutionary rate of the 16S rRNA gene among these genera seems to be different and it is specifically related to the evolution within this clade (Chelo et al. 2007). Although the 16S rRNA gene has been used in the constitution of the present taxonomy, the validity of phylogenetic inferences drawn based on this gene is limited (Chelo et al. 2007).

1.9.5 Summary

The importance of the genus *Weissella* in food fermentations has been established by worldwide studies. Since the members from this genus are widely spread in various environmental niches, it would be of interest to study the biodiversity and evolution of the isolates in detail. Depending on the discriminatory power of the methods used, different phylogenetic inferences may be resulted. Therefore, further systematic studies using advanced molecular typing tools such as Mutilocus Sequence Typing (MLST) are anticipated.

References

Alegría Á, Delgado S, Flórez AB, Mayo B. Identification, typing and functional characterisation of *Leuconostoc* spp. strains from traditional, starter-free cheeses. Dairy Sci Technol. 2013;93 (6):657–73.

Amari M, Laguerre S, Vuillemin M, Robert H, Loux V, Klopp C, et al. Genome sequence of *Weissella confusa* LBAE C39-2, isolated from a wheat sourdough. J Bacteriol. 2012;194 (6):1608–9. doi:10.1128/JB.06788-11.

Ampe F, ben Omar N, Moizan C, Wacher C, Guyot JP. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Appl Environ Microbiol. 1999;65(12):5464–73.

Andrewes F, Horder T. A study of the streptococci pathogenic for man. Lancet. 1906;168 (4334):775–83.

Arahal DR, Sanchez E, Macian MC, Garay E. Value of recN sequences for species identification and as a phylogenetic marker within the family "*Leuconostocaceae*". Int Microbiol. 2008;11 (1):33–9.

Back W. Zur taxonomie der gattung Pediococcus. Brauwissenschaft. 1978;31:237–50.

Bain JM, Tavanti A, Davidson AD, Jacobsen MD, Shaw D, Gow NA, et al. Multilocus sequence typing of the pathogenic fungus *Aspergillus fumigatus*. J Clin Microbiol. 2007;45(5):1469–77.

Balcke J. Über faurigen Geruch des Bieres. Wochenschrift fur Brauerei. 1884;1:181–4.

Bandelt H, Dress A. A canonical decomposition theory for metrics on a finite set. Adv Math. 1992;92(1):47–105.

Barnes EM, Impey C, Stevens B, Peel J. *Streptococcus pleomorphus* sp. nov.: an anaerobic streptococcus isolated mainly from the caeca of birds. J Gen Microbiol. 1977;102(1):45–53.

Barrangou R, Yoon SS, Breidt F Jr, Fleming HP, Klaenhammer TR. Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. Appl Environ Microbiol. 2002;68(6):2877–84.

Barros RR, Carvalho MG, Peralta JM, Facklam RR, Teixeira LM. Phenotypic and genotypic characterization of *Pediococcus* strains isolated from human clinical sources. J Clin Microbiol. 2001;39(4):1241–6. doi:10.1128/JCM.39.4.1241-1246.2001.

Barsotti O, Décoret D, Renaud FNR. Identification of *streptococcus mitis* group species by RFLP of the PCR-amplified 16S-23S rDNA intergenic spacer. Res Microbiol. 2002;153(10):687–91. doi:http://dx.doi.org/10.1016/S0923-2508(02)01382-7.

Bartish IV, Garkava LP, Rumpunen K, Nybom H. Phylogenetic relationships and differentiation among and within populations of Chaenomeles Lindl. (Rosaceae) estimated with RAPDs and isozymes. Theor Appl Genet. 2000;101(4):554–63.

Beighton D, Hardie J, Whiley R. A scheme for the identification of viridans streptococci. J Med Microbiol. 1991;35(6):367–72.

Beijerinck M. Sur les ferments lactiques de l'industrie. Arch Neerl Sci. 1901;6:212–43.

Benomar N, Abriouel H, Lee H, Cho GS, Huch M, Pulido RP, et al. Genome sequence of *Weissella thailandensis* fsh4-2. J Bacteriol. 2011;193(20):5868. doi:10.1128/JB.05883-11.

Bentley RW, Leigh JA, Collins MD. Intrageneric structure of *streptococcus* based on comparative analysis of small-subunit rRNA sequences. Int J Syst Bacteriol. 1991;41 (4):487–94.

Bergey DH, Buchanan RE, Gibbons NE. Bergey's manual of determinative bacteriology. 8th ed. Baltimore: Williams & Wilkins Co.; 1974.

Betzl D, Ludwig W, Schleifer KH. Identification of lactococci and enterococci by colony hybridization with 23S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol. 1990;56(9):2927–9.

Biavati B, Mattarelli P. Genus I. *Bifidobacterium* Oral-Jansen 1924. In: Goodfellow M, Kampfer P, Busse H-J, Suzuki K-I, Ludwig W, Whitman W, editors. Bergey's manual of systematic bacteriology. 2nd ed. Berlin: Springer; 2012. p. 171–206.

Biavati B, Mattarelli P. *Bifidobacterium ruminantium* sp. nov. and *Bifidobacterium merycicum* sp. nov. from the rumens of cattle. Int J Syst Bacteriol. 1991;41(1):163–8.

Biavati B, Mattarelli P. The family *Bifidobacteriaceae*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebandt E, editors. The prokaryotes. 3rd ed. New York: Springer; 2006. p. 322–82.

Biavati B, Scardovi V, Moore W. Electrophoretic patterns of proteins in the genus *Bifidobacterium* and proposal of four new species. Int J Syst Bacteriol. 1982;32(3):358–73.

Billroth T. Untersuchungen über die Vegetationsformen von Coccobacteria septica und den Antheil, welchen sie an der Entstehung und Verbreitung der accidentellen Wundkrankheiten haben. Berlin: Georg Reimer; 1874.

Bjorkroth KJ, Vandamme P, Korkeala HJ. Identification and characterization of *Leuconostoc carnosum*, associated with production and spoilage of vacuum-packaged, sliced, cooked ham. Appl Environ Microbiol. 1998;64(9):3313–9.

Bjorkroth KJ, Geisen R, Schillinger U, Weiss N, De Vos P, Holzapfel WH, et al. Characterization of *Leuconostoc gasicomitatum* sp. nov., associated with spoiled raw tomato-marinated broiler meat strips packaged under modified-atmosphere conditions. Appl Environ Microbiol. 2000;66(9):3764–72.

Bjorkroth KJ, Schillinger U, Geisen R, Weiss N, Hoste B, Holzapfel WH, et al. Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., detected in food and clinical samples. Int J Syst Evol Microbiol. 2002;52(Pt 1):141–8.

Blaiotta G, Fusco V, Ercolini D, Aponte M, Pepe O, Villani F. *Lactobacillus* strain diversity based on partial *hsp*60 gene sequences and design of PCR-restriction fragment length

polymorphism assays for species identification and differentiation. Appl Environ Microbiol. 2008;74(1):208–15.

Boers SA, van der Reijden WA, Jansen R. High-throughput multilocus sequence typing: bringing molecular typing to the next level. PLoS One. 2012;7(7):e39630.

Bolotin A, Mauger S, Malarme K, Ehrlich SD, Sorokin A. Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. In: Konings W, Kuipers O, Huis IH, editors. Lactic acid bacteria: genetics, metabolism and applications. New York: Springer; 1999. p. 27–76.

Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, et al. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. 2001;11(5):731–53.

Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, Kulakauskas S, et al. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol. 2004;22(12):1554–8. doi:10.1038/nbt1034.

Bonora MG, Ligozzi M, De Fatima M, Bragagnolo L, Goglio A, Guazzotti GC, et al. Vancomycin-resistant *Enterococcus faecium* isolates causing hospital outbreaks in northern Italy belong to the multilocus sequence typing C1 lineage. Microb Drug Resist. 2004;10 (2):114–23. doi:10.1089/1076629041310046.

Bottacini F, Medini D, Pavesi A, Turroni F, Foroni E, Riley D, et al. Comparative genomics of the genus *Bifidobacterium*. Microbiology. 2010;156(Pt 11):3243–54. doi:10.1099/mic.0.039545-0.

Brown JH. The use of blood agar for the study of streptococci, vol. 9. New York: The Rockefeller Institute for Medical Research; 1919.

Buhnik-Rosenblau K, Matsko-Efimov V, Jung M, Shin H, Danin-Poleg Y, Kashi Y. Indication for Co-evolution of *Lactobacillus johnsonii* with its hosts. BMC Microbiol. 2012;12:149. doi:10.1186/1471-2180-12-149.

Bui TP, Kim YJ, In JG, Yang DC. *Lactobacillus koreensis* sp. nov., isolated from the traditional Korean food kimchi. Int J Syst Evol Microbiol. 2011;61(Pt 4):772–6. doi:10.1099/ijs.0.021386-0.

Cai Y, Benno Y, Nakase T, Oh TK. Specific probiotic characterization of *Weissella hellenica* DS-12 isolated from flounder intestine. J Gen Appl Microbiol. 1998;44(5):311–6.

Cai H, Rodriguez BT, Zhang W, Broadbent JR, Steele JL. Genotypic and phenotypic characterization of *Lactobacillus casei* strains isolated from different ecological niches suggests frequent recombination and niche specificity. Microbiology. 2007;153(Pt 8):2655–65.

Cai H, Thompson R, Budinich MF, Broadbent JR, Steele JL. Genome sequence and comparative genome analysis of *Lactobacillus casei*: insights into their niche-associated evolution. Genome Biol Evol. 2009;1:239–57. doi:10.1093/gbe/evp019.

Cai Y, Yang J, Pang H, Kitahara K. *Lactococcus fujiensis* sp. nov., a lactic acid bacterium isolated from vegetable matter. Int J Syst Evol Microbiol. 2011;61(7):1590–4.

Cai Y, Pang H, Kitahara M, Ohkuma M. *Lactobacillus nasuensis* sp. nov., a lactic acid bacterium isolated from silage, and emended description of the genus *Lactobacillus*. Int J Syst Evol Microbiol. 2012;62(Pt 5):1140–4. doi:10.1099/ijs.0.031781-0.

Calmin G, Lefort F, Belbahri L. Multi-loci sequence typing (MLST) for two lacto-acid bacteria (LAB) species: *Pediococcus parvulus* and *P. damnosus*. Mol Biotechnol. 2008;40(2):170–9. doi:10.1007/s12033-008-9073-4.

Camargo IL, Gilmore MS, Darini AL. Multilocus sequence typing and analysis of putative virulence factors in vancomycin-resistant and vancomycin-sensitive *Enterococcus faecium* isolates from Brazil. Clin Microbiol Infect. 2006;12(11):1123–30. doi:10.1111/j.1469-0691.2006.01496.x.

Carr FJ, Chill D, Maida N. The lactic acid bacteria: a literature survey. Crit Rev Microbiol. 2002;28(4):281–370. doi:10.1080/1040-840291046759.

Chaillou S, Lucquin I, Najjari A, Zagorec M, Champomier-Verges MC. Population genetics of *Lactobacillus sakei* reveals three lineages with distinct evolutionary histories. PLoS One. 2013;8(9):e73253. doi:10.1371/journal.pone.0073253.

Chang JY, Chang HC. Identification of a replicon from pCC3, a cryptic plasmid from *Leuconostoc citreum* C4 derived from kimchi, and development of a new host-vector system. Biotechnol Lett. 2009;31(5):685–96.
Chao SH, Kudo Y, Tsai YC, Watanabe K. *Lactobacillus futsaii* sp. nov., isolated from fu-tsai and suan-tsai, traditional Taiwanese fermented mustard products. Int J Syst Evol Microbiol. 2012;62(Pt 3):489–94. doi:10.1099/ijs.0.030619-0.
Chatellier S, Ramanantsoa C, Harriau P, Rolland K, Rosenau A, Quentin R. Characterization of *Streptococcus agalactiae* strains by randomly amplified polymorphic DNA analysis. J Clin Microbiol. 1997;35(10):2573–9.
Chavagnat F, Haueter M, Jimeno J, Casey MG. Comparison of partial tuf gene sequences for the identification of lactobacilli. FEMS Microbiol Lett. 2002;217(2):177–83.
Chelo IM, Ze-Ze L, Tenreiro R. Congruence of evolutionary relationships inside the *Leuconostoc-Oenococcus-Weissella* clade assessed by phylogenetic analysis of the 16S rRNA gene, dnaA, gyrB, rpoC and dnaK. Int J Syst Evol Microbiol. 2007;57(Pt 2):276–86. doi:10.1099/ijs.0.64468-0.
Chen YS, Chang CH, Pan SF, Wang LT, Chang YC, Wu HC, et al. *Lactococcus taiwanensis* sp. nov., a novel lactic acid bacterium isolated from fresh cummingcordia. Int J Syst Evol Microbiol. 2012;. doi:10.1099/ijs.0.045757-0.
Chenoll E, Carmen Macian M, Aznar R. *Lactobacillus tucceti* sp. nov., a new lactic acid bacterium isolated from sausage. Syst Appl Microbiol. 2006;29(5):389–95. doi:10.1016/j.syapm.2005.09.006.
Chenoll E, Macian MC, Aznar R. Identification of *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* by rDNA-based techniques. Syst Appl Microbiol. 2003;26(4):546–56. doi:10.1078/072320203770865855.
Chester FD. A manual of determinative bacteriology. New York: The Macmillan Co.; 1901.
Cho JC, Tiedje JM. Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. Appl Environ Microbiol. 2001;67(8):3677–82.
Cho SL, Nam SW, Yoon JH, Lee JS, Sukhoom A, Kim W. *Lactococcus chungangensis* sp. nov., a lactic acid bacterium isolated from activated sludge foam. Int J Syst Evol Microbiol. 2008;58(8):1844–9.
Choi HJ, Cheigh CI, Kim SB, Lee JC, Lee DW, Choi SW, et al. *Weissella kimchii* sp. nov., a novel lactic acid bacterium from kimchi. Int J Syst Evol Microbiol. 2002;52(Pt 2):507–11.
Cibik R, Lepage E, Tailliez P. Molecular diversity of *Leuconostoc mesenteroides* and *Leuconostoc citreum* isolated from traditional french cheeses as revealed by RAPD fingerprinting, 16S rDNA sequencing and 16S rDNA fragment amplification. Syst Appl Microbiol. 2000;23(2):267–78.
Claesson MJ, van Sinderen D, O'Toole PW. *Lactobacillus* phylogenomics–towards a reclassification of the genus. Int J Syst Evol Microbiol. 2008;58(Pt 12):2945–54. doi:10.1099/ijs.0.65848-0.
Clark NC, Teixeira LM, Facklam RR, Tenover FC. Detection and differentiation of vanC-1, vanC-2, and vanC-3 glycopeptide resistance genes in enterococci. J Clin Microbiol. 1998;36(8):2294–7.
Clarke JK. On the bacterial factor in the aetiology of dental caries. Br J Exp Pathol. 1924;5(3):141.
Claussen NH. Études sur les bactéries dites sarcines et sur les maladies qu'elles provoquent dans la bière. Compte Rendu des Travaux du Laboratoire de Carlsberg. 1903;6:64–83.
Cocconcelli PS, Porro D, Galandini S, Senini L. Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. Lett Appl Microbiol. 1995;21(6):376–9.
Collins MD, Lundström T, Welinder-Olsson C, Hansson I, Wattle O, Hudson RA et al. *Streptococcus devriesei* sp. nov., from Equine Teeth. Syst Appl Microbiol. 2004;27(2):146–50. doi:http://dx.doi.org/10.1078/072320204322881754.
Collins MD, Wallbanks S. Comparative sequence analyses of the 16S rRNA genes of *Lactobacillus minutus*, *Lactobacillus rimae* and *Streptococcus parvulus*: proposal for the creation of a new genus Atopobium. FEMS Microbiol Lett. 1992;74(2–3):235–40.

Collins M, Farrow J, Katic V, Kandler O. Taxonomic studies on streptococci of serological groups E, P, U and V: Description of *Streptococcus porcinus* sp. nov. Syst Appl Microbiol. 1984a;5(3):402–13.

Collins M, Jones D, Farrow J, Kilpper-Balz R, Schleifer K. *Enterococcus avium* nom. rev., comb. nov.; *E. casseliflavus* nom. rev., comb. nov.; *E. durans* nom. rev., comb. nov.; *E. gallinarum* comb. nov.; and *E. malodoratus* sp. nov. Int J Syst Bacteriol. 1984b;34(2):220–3.

Collins M, Rodrigues U, Ash C, Aguirre M, Farrow J, Martinez-Murcia A, et al. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol Lett. 1991;77(1):5–12.

Collins MD, Samelis J, Metaxopoulos J, Wallbanks S. Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus Weissella for the *Leuconostoc paramesenteroides* group of species. J Appl Bacteriol. 1993;75(6):595–603.

Colman G, Parker M, Duerden B. Streptococcus and Lactobacillus. In: Parker MT, Duerden BI, editors. Topley & Wilson's principles of bacteriology, virology and immunity. 8th ed. London: Edward Arnold; 1990. p. 119–59.

Colman G, Williams R. The cell walls of streptococci. J Gen Microbiol. 1965;41(3):375–87.

Colman G, Williams R. Taxonomy of some human viridans streptococci. In: Wannamaker L, Matsen J, editors. Streptococci and streptococcal diseases: recognition, understanding, and management. New York: Academic Press; 1972. p. 281–99.

Cooke RD, Twiddy DR, Alan Reilly PJ. Lactic-acid fermentation as a low-cost means of food preservation in tropical countries. FEMS Microbiol Lett. 1987;46(3):369–79.

Coykendall AL, Wesbecher PM, Gustafson KB. "*Streptococcus milleri*" *Streptococcus constellatus*, and *Streptococcus intermedius* are later synonyms of *Streptococcus anginosus*. Int J Syst Bacteriol. 1987;37(3):222–8.

De Bruyne K, Schillinger U, Caroline L, Boehringer B, Cleenwerck I, Vancanneyt M, et al. *Leuconostoc holzapfelii* sp. nov., isolated from Ethiopian coffee fermentation and assessment of sequence analysis of housekeeping genes for delineation of Leuconostoc species. Int J Syst Evol Microbiol. 2007;57(12):2952–9.

De Bruyne K, Camu N, Lefebvre K, De Vuyst L, Vandamme P. *Weissella ghanensis* sp. nov., isolated from a Ghanaian cocoa fermentation. Int J Syst Evol Microbiol. 2008a;58(Pt 12):2721–5. doi:10.1099/ijs.0.65853-0.

De Bruyne K, Franz CM, Vancanneyt M, Schillinger U, Mozzi F, de Valdez GF, et al. *Pediococcus argentinicus* sp. nov. from Argentinean fermented wheat flour and identification of Pediococcus species by pheS, rpoA and atpA sequence analysis. Int J Syst Evol Microbiol. 2008b;58(Pt 12):2909–16. doi:10.1099/ijs.0.65833-0.

De Bruyne K, Camu N, De Vuyst L, Vandamme P. *Weissella fabaria* sp. nov., from a Ghanaian cocoa fermentation. Int J Syst Evol Microbiol. 2009;60(Pt 9):1999–2005. doi:10.1099/ijs.0.019323-0.

De Las Rivas B, Marcobal A, Muñoz R. Allelic diversity and population structure in *Oenococcus oeni* as determined from sequence analysis of housekeeping genes. Appl Environ Microbiol. 2004;70(12):7210–9.

de Las Rivas B, Marcobal A, Munoz R. Development of a multilocus sequence typing method for analysis of *Lactobacillus plantarum* strains. Microbiology. 2006;152(Pt 1):85–93.

Deletoile A, Passet V, Aires J, Chambaud I, Butel MJ, Smokvina T, et al. Species delineation and clonal diversity in four *Bifidobacterium* species as revealed by multilocus sequencing. Res Microbiol. 2010;161(2):82–90. doi:10.1016/j.resmic.2009.12.006.

Delorme C, Bartholini C, Bolotine A, Ehrlich SD, Renault P. Emergence of a cell wall protease in the *Streptococcus thermophilus* population. Appl Environ Microbiol. 2010;76(2):451–60. doi:10.1128/AEM.01018-09.

Descheemaeker P, Lammens C, Pot B, Vandamme P, Goossens H. Evaluation of arbitrarily primed PCR analysis and pulsed-field gel electrophoresis of large genomic DNA fragments for identification of enterococci important in human medicine. Int J Syst Bacteriol. 1997;47 (2):555–61.

Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811–8. doi:10.1038/nature06245.

Deveau H, Moineau S. Technical note: use of RFLP to characterize *Lactococcus lactis* Strains producing exopolysaccharides. J Dairy Sci. 2003;86(4):1472–5. doi:http://dx.doi.org/10.3168/jds.S0022-0302(03)73731-X.

Devriese L, Van de Kerckhove A, Kilpper-Bälz R, Schleifer K. Characterization and identification of Enterococcus species isolated from the intestines of animals. Int J Syst Bacteriol. 1987;37(3):257–9.

Devriese L, Pot B. The genus Enterococcus. In: Wood B, Holzapfel W, editors. The genera of lactic acid bacteria. London: Blackie Academic & Professional; 1995. p. 327–67.

Devriese LA, Pot B, Vandamme P, Kersters K, Collins MD, Alvarez N, et al. *Streptococcus hyovaginalis* sp. nov. and *Streptococcus thoraltensis* sp. nov., from the genital tract of sows. Int J Syst Bacteriol. 1997;47(4):1073–7.

Devriese LA, Vancanneyt M, Descheemaeker P, Baele M, Van Landuyt HW, Gordts B, et al. Differentiation and identification of *Enterococcus durans*, E. hirae and E. villorum. J Appl Microbiol. 2002;92(5):821–7.

Devriese L, Baele M, Butaye P. The genus *Enterococcus*: taxonomy. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebandt E, editors. The prokaryotes. 3rd ed. New York: Springer; 2006. p. 163–74.

Di Cagno R, De Angelis M, Limitone A, Minervini F, Carnevali P, Corsetti A, et al. Glucan and fructan production by sourdough *Weissella cibaria* and *Lactobacillus plantarum*. J Agric Food Chem. 2006;54(26):9873–81. doi:10.1021/jf061393+.

Diancourt L, Passet V, Chervaux C, Garault P, Smokvina T, Brisse S. Multilocus sequence typing of *Lactobacillus casei* reveals a clonal population structure with low levels of homologous recombination. Appl Environ Microbiol. 2007;73(20):6601–11. doi:10.1128/AEM.01095-07.

Dicks LMT, Dellaglio F, Collins MD. Proposal To Reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. Int J Syst Evol Microbiol. 1995;45(2):395–7.

Dimitrov Z. Development of strain discriminative amplified fragment length polymorpfic DNA for Bifidobacteria. Design of strain-specific markers. Mol Biol. 2011;6:07.

Dobson CM, Deneer H, Lee S, Hemmingsen S, Glaze S, Ziola B. Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer. Int J Syst Evol Microbiol. 2002;52(Pt 6):2003–10.

Dong X, Cheng G, Jian W. Simultaneous Identification of Five Bifidobacterium species isolated from human beings using multiple PCR primers. Syst Appl Microbiol. 2000;23(3):386–90.

Douillard FP, Ribbera A, Kant R, Pietila TE, Jarvinen HM, Messing M, et al. Comparative genomic and functional analysis of 100 *Lactobacillus rhamnosus* strains and their comparison with strain GG. PLoS Genet. 2013;9(8):e1003683. doi:10.1371/journal.pgen.1003683.

Duh R-W, Singh KV, Malathum K, Murray BE. In vitro activity of 19 antimicrobial agents against enterococci from healthy subjects and hospitalized patients and use of an ace gene probe from *Enterococcus faecalis* for species identification. Microb Drug Resist. 2001;7(1):39–46.

Ehrmann MA, Freiding S, Vogel RF. *Leuconostoc palmae* sp. nov., a novel lactic acid bacterium isolated from palm wine. Int J Syst Evol Microbiol. 2009;59(5):943–7.

Endo A, Okada S. Reclassification of the genus *Leuconostoc* and proposals of *Fructobacillus fructosus* gen. nov., comb. nov., *Fructobacillus durionis* comb. nov., *Fructobacillus ficulneus* comb. nov. and *Fructobacillus pseudoficulneus* comb. nov. Int J Syst Evol Microbiol. 2008;58(9):2195–205.

Endo A, Futagawa-Endo Y, Schumann P, Pukall R, Dicks LM. *Bifidobacterium reuteri* sp. nov., *Bifidobacterium callitrichos* sp. nov., *Bifidobacterium saguini* sp. nov., *Bifidobacterium stellenboschense* sp. nov. and *Bifidobacterium biavatii* sp. nov. isolated from faeces of common marmoset (Callithrix jacchus) and red-handed tamarin (Saguinus midas). Syst Appl Microbiol. 2012;35(2):92–7. doi:10.1016/j.syapm.2011.11.006.

Ennahar S, Cai Y. Genetic evidence that *Weissella kimchii* Choi et al. 2002 is a later heterotypic synonym of *Weissella cibaria* Bjorkroth et al. 2002. Int J Syst Evol Microbiol. 2004;54(Pt 2):463–5.

Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology. 1998;144 (11):3049–60.

Enright MC, Spratt BG. Multilocus sequence typing. Trends Microbiol. 1999;7(12):482–7.

Enright MC, Spratt BG, Kalia A, Cross JH, Bessen DE. Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between emm type and clone. Infect Immun. 2001;69(4):2416–27.

Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc Natl Acad Sci U S A. 2002;99(11):7687–92.

Euzeby JP. Necessary corrections according to judicial opinions 16, 48 and 52. Int J Syst Bacteriol. 1998;48(Pt 2):613.

Ezaki T, Li N, Hashimoto Y, Miura H, Yamamoto H. 16S ribosomal DNA sequences of anaerobic cocci and proposal of *Ruminococcus hansenii* comb. nov. and *Ruminococcus productus* comb. nov. Int J Syst Bacteriol. 1994;44(1):130–6.

Facklam R, Wilkinson H. The family *Streptococcaceae* (medical aspects). Prokaryotes. 1981;2:1572–97.

Facklam RR, Carvalho M-G, Teixeira LM. History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci. In: Gilmore MS, editor. The enterococci: pathogenesis, molecular biology, and antibiotic resistance. Washington: ASM Press; 2002. p. 1–54.

Farrow J, Collins M. Taxonomic studies on streptococci of serological groups C, G and L and possibly related taxa. Syst Appl Microbiol. 1984a;5(4):483–93.

Farrow J, Collins M. DNA base composition, DNA-DNA homology and long-chain fatty acid studies on *Streptococcus thermophilus* and *Streptococcus salivarius*. J Gen Microbiol. 1984b;130(2):357–62.

Farrow JAE, Kruze J, Phillips BA, Bramley AJ, Collins MD. Taxonomic Studies on *Streptococcus bovis* and *Streptococcus equinus*: description of *Streptococcus alactolyticus* sp. nov. and *Streptococcus saccharolyticus* sp. nov. Syst Appl Microbiol. 1984;5(4):467–482. doi:http://dx.doi.org/10.1016/S0723-2020(84)80004-1.

Farrow JA, Facklam RR, Collins MD. Nucleic acid homologies of some vancomycin-resistant *leuconostocs* and description of *Leuconostoc citreum* sp. nov. and *Leuconostoc pseudomes-enteroides* sp. nov. Int J Syst Bacteriol. 1989;39(3):279–83.

Feil EJ, Smith JM, Enright MC, Spratt BG. Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. Genetics. 2000;154 (4):1439–50.

Feil EJ, Cooper JE, Grundmann H, Robinson DA, Enright MC, Berendt T, et al. How clonal is *Staphylococcus aureus*? J Bacteriol. 2003;185(11):3307–16.

Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186(5):1518–30.

Felis GE, Dellaglio F. Taxonomy of Lactobacilli and Bifidobacteria. Curr Issues Intest Microbiol. 2007;8(2):44–61.

Felis GE, Dellaglio F, Mizzi L, Torriani S. Comparative sequence analysis of a recA gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group. Int J Syst Evol Microbiol. 2001;51(Pt 6):2113–7.

Figueiredo HC, Costa FA, Leal CA, Carvalho-Castro GA, Leite RC. *Weissella* sp. outbreaks in commercial rainbow trout (Oncorhynchus mykiss) farms in Brazil. Vet Microbiol. 2011;156 (3–4):359–66. doi:10.1016/j.vetmic.2011.11.008.

Foschino R, Nucera D, Volponi G, Picozzi C, Ortoffi M, Bottero M. Comparison of *Lactococcus garvieae* strains isolated in northern Italy from dairy products and fishes through molecular typing. J Appl Microbiol. 2008;105(3):652–62.

Foulquie Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of enterococci in food and health. Int J Food Microbiol. 2006;106(1):1–24. doi:10.1016/j.ijfoodmicro.2005.06.026.

Franz CM, Vancanneyt M, Vandemeulebroecke K, De Wachter M, Cleenwerck I, Hoste B, et al. *Pediococcus stilesii* sp. nov., isolated from maize grains. Int J Syst Evol Microbiol. 2006;56 (Pt 2):329–33. doi:10.1099/ijs.0.63944-0.

Friedland I, Snipelisky M, Khoosal M. Meningitis in a neonate caused by *Leuconostoc* sp. J Clin Microbiol. 1990;28(9):2125–6.

Fuller JD, Bast DJ, Nizet V, Low DE, de Azavedo JC. *Streptococcus iniae* virulence is associated with a distinct genetic profile. Infect Immun. 2001;69(4):1994–2000.

Garvie EI. The growth factor and amino acid requirements of species of the genus *Leuconostoc*, including *Leuconostoc paramesenteroides* (sp. nov.) and *Leuconostoc oenos*. J Gen Microbiol. 1967;48(3):439–47.

Garvie EI. NOTES: *Leuconostoc mesenteroides* subsp. *cremoris* (Knudsen and Sørensen) comb. nov. and *Leuconostoc mesenteroides* subsp. *dextranicum* (Beijerinck) comb. nov. Int J Syst Bacteriol. 1983;33(1):118–9.

Garvie EI. Separation of Species of the Genus *Leuconostoc* and differentiation of the *leuconostocs* from other lactic acid bacteria. Methods Microbiol. 1984;16:147–78.

Gaudu P, Vido K, Cesselin B, Kulakauskas S, Tremblay J, Rezaïki L, et al. Respiration capacity and consequences in *Lactococcus lactis*. Antonie Van Leeuwenhoek. 2002;82(1–4):263–9.

Glazunova OO, Raoult D, Roux V. Partial sequence comparison of the rpoB, sodA, groEL and gyrB genes within the genus *Streptococcus*. Int J Syst Evol Microbiol. 2009;59(9):2317–22.

Glick MC, Sall T, Zilliken F, Mudd S. Morphological changes of *Lactobacillus bifidus* var. pennsylvanicus produced by a cell-wall precursor. Biochim Biophys Acta. 1960;37:361–3.

Goh SH, Facklam RR, Chang M, Hill JE, Tyrrell GJ, Burns EC, et al. Identification of *Enterococcus* species and phenotypically similar *Lactococcus* and *Vagococcus* species by reverse checkerboard hybridization to chaperonin 60 gene sequences. J Clin Microbiol. 2000;38(11):3953–9.

Gu CT, Wang F, Li CY, Liu F, Huo GC. *Leuconostoc mesenteroides* subsp. *suionicum* subsp. nov. Int J Syst Evol Microbiol. 2012;62(7):1548–51.

Gunther HL, White HR. The cultural and physiological characters of the pediococci. J Gen Microbiol. 1961;26:185–97.

Haakensen M, Dobson CM, Hill JE, Ziola B. Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*. Int J Syst Evol Microbiol. 2009;59(Pt 3):615–21. doi:10.1099/ijs.0.65779-0.

Häggström MH. Effects of growth conditions on the activities of superoxide dismutase and NADH-oxidase/NADH-peroxidase in *Streptococcus lactis*. Curr Microbiol. 1984;10(6):345–51.

Hammes WP, Hertel C. The genera Lactobacillus and Carnobacterium. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebandt E, editors. The prokaryotes. 3rd ed. New York: Springer; 2006. p. 319–403.

Hammes W, Hertel C. Genus I. *Lactobacillus Beijerink* 1901. In: De-Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F et al., editors. Bergey's manual of systematic bacteriology. 2nd ed. Berlin: Springer; 2009. p. 465–510.

Hammes WP, Vogel RF. The genus *Lactobacillus*. In: Wood B, Holzapfel W, editors. The genera of lactic acid bacteria. London: Blackie Academic & Professional; 1995. p. 19–54.

Hardie J, Marsh P, editors. Streptococci and the human oral flora. Soc Appl Bacteriol Symp Ser. 1978;7:157–206.

Hardie JM, Whiley RA. The genus Streptococcus—Oral. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebandt E, editors. The prokaryotes. New York: Springer; 2006. p. 76–107.

Haubold B, Hudson RR. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage analysis. Bioinformatics. 2000;16(9):847–8.

Hillman J, Andrews S, Painter S, Stashenko P. Adaptive changes in a strain of *Streptococcus mutans* during colonisation of the human oral cavity. Microb Ecol Health Dis. 1989;2 (4):231–9.

Holzapfel WH, Franz CMAP, Ludwig W, Dicks LMT. Genus I. Bacillus Cohn 1872. In: De-Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F et al., editors. Bergey's manual of systematic bacteriology. Berlin: Springer; 2009. p. 21–128.

Holzapfel WH, Franz CMAP, Ludwig W, Dicks LMT. Genus III. *Pediococcus* Claussen 1903. In: De-Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F et al., editors. Bergey's manual of systematic bacteriology. 2nd ed. Berlin: Springer; 2009. p. 513–32.

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J, Schillinger U. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr. 2001;73(2 Suppl):365S–73S.

Holzapfel WH, Franz CMAP, Ludwig W, Back W, Dicks LMT. The genera *Pedicoccus* and *Tetragenococcus*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebandt E, editors. The prokaryotes. 3rd ed. New York: Springer; 2006. p. 229–66.

Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, et al. Multilocus sequence typing scheme for *Enterococcus faecium*. J Clin Microbiol. 2002;40(6):1963–71.

Horvath P, Coute-Monvoisin AC, Romero DA, Boyaval P, Fremaux C, Barrangou R. Comparative analysis of CRISPR loci in lactic acid bacteria genomes. Int J Food Microbiol. 2009;131(1):62–70. doi:10.1016/j.ijfoodmicro.2008.05.030.

Hoshino T, Fujiwara T, Kilian M. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. J Clin Microbiol. 2005;43 (12):6073–85.

Hucker GJ, Pederson CS. A study of the physiology and classification of the genus *Leuconostoc*. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. 1931;2(85):65–114.

Husain I, Poupard JA, Norris RF. Influence of nutrition on the morphology of a strain of *Bifidobacterium bifidum*. J Bacteriol. 1972;111(3):841–4.

Huson DH. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics. 1998;14 (1):68–73.

Ilse S, Roel VD, Gino V, Luc DV, Luca S, Peter V et al. Polyphasic taxonomic characterization of *Lactobacillus rossiae* isolates from Belgian and Italian sourdoughs reveals intraspecific heterogeneity. Syst Appl Microbiol. 2009;32:151–6.

Itoh Y, Kawamura Y, Kasai H, Shah MM, Nhung PH, Yamada M et al. *dna*J and *gyr*B gene sequence relationship among species and strains of genus *Streptococcus*. Syst Appl Microbiol. 2006;29(5):368–74. doi:http://dx.doi.org/10.1016/j.syapm.2005.12.003.

Jackson CR, Fedorka-Cray PJ, Barrett JB. Use of a genus-and species-specific multiplex PCR for identification of enterococci. J Clin Microbiol. 2004;42(8):3558–65.

Jacobs JA, Tjhie JH, Smeets MG, Schot CS, Schouls LM. Genotyping by amplified fragment length polymorphism analysis reveals persistence and recurrence of infection with *Streptococcus anginosus* group organisms. J Clin Microbiol. 2003;41(7):2862–6.

Jang J, Kim B, Lee J, Kim J, Jeong G, Han H. Identification of Weissella species by the genus-specific amplified ribosomal DNA restriction analysis. FEMS Microbiol Lett. 2002;212 (1):29–34. doi:S0378109702006808.

Jang J, Kim B, Lee J, Han H. A rapid method for identification of typical *Leuconostoc* species by 16S rDNA PCR-RFLP analysis. J Microbiol Methods. 2003;55(1):295–302.

Jayarao B, Dore J, Oliver S. Restriction fragment length polymorphism analysis of 16S ribosomal DNA of Streptococcus and Enterococcus species of bovine origin. J Clin Microbiol. 1992;30 (9):2235–40.

Jensen A, Hoshino T, Kilian M. Taxonomy of the Anginosus group of the genus Streptococcus and description of *Streptococcus anginosus* subsp. whileyi subsp. nov. and *Streptococcus constellatus* subsp. viborgensis subsp. nov. Int J Syst Evol Microbiol. 2012;63:2506–19.

Jeong SJ, Park JY, Lee HJ, Kim JH. Characterization of pFMBL1, a small cryptic plasmid isolated from *Leuconostoc mesenteroides* SY2. Plasmid. 2007;57(3):314–23.

Jian W, Zhu L, Dong X. New approach to phylogenetic analysis of the genus Bifidobacterium based on partial HSP60 gene sequences. Int J Syst Evol Microbiol. 2001;51(Pt 5):1633–8.

Jolley KA, Feil EJ, Chan MS, Maiden MC. Sequence type analysis and recombinational tests (START). Bioinformatics. 2001;17(12):1230–1.

Jones D. Composition and differentiation of the genus *Streptococcus*. Soc Appl Bacteriol Symp Ser. 1978;7:1–49.

Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan M-S, Kunst F, et al. Multilocus sequence typing system for group B Streptococcus. J Clin Microbiol. 2003;41(6):2530–6.

Kahala M, Maki M, Lehtovaara A, Tapanainen JM, Katiska R, Juuruskorpi M, et al. Characterization of starter lactic acid bacteria from the Finnish fermented milk product viili. J Appl Microbiol. 2008;105(6):1929–38.

Kang MS, Na HS, Oh JS. Coaggregation ability of Weissella cibaria isolates with Fusobacterium nucleatum and their adhesiveness to epithelial cells. FEMS Microbiol Lett. 2005;253(2):323–9. doi:10.1016/j.femsle.2005.10.002.

Kang MS, Chung J, Kim SM, Yang KH, Oh JS. Effect of Weissella cibaria isolates on the formation of *Streptococcus mutans* biofilm. Caries Res. 2006;40(5):418–25. doi:10.1159/000094288.

Kariyama R, Mitsuhata R, Chow JW, Clewell DB, Kumon H. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J Clin Microbiol. 2000;38(8):3092–5.

Kawamura Y, Hou X-G, Sultana F, Miura H, Ezaki T. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Bacteriol. 1995;45(2):406–8.

Kawamura Y, Whiley RA, Shu S-E, Ezaki T, Hardie JM. Genetic approaches to the identification of the mitis group within the genus Streptococcus. Microbiology. 1999;145(9):2605–13.

Kelly WJ, Asmundson RV, Harrison GL, Huang CM. Differentiation of dextran-producing Leuconostoc strains from fermented rice cake (puto) using pulsed-field gel electrophoresis. Int J Food Microbiol. 1995;26(3):345–52.

Kelly WJ, Davey GP, Ward LJ. Characterization of lactococci isolated from minimally processed fresh fruit and vegetables. Int J Food Microbiol. 1998;45(2):85–92.

Kikuchi K, Enari T, Totsuka K, Shimizu K. Comparison of phenotypic characteristics, DNA-DNA hybridization results, and results with a commercial rapid biochemical and enzymatic reaction system for identification of viridans group streptococci. J Clin Microbiol. 1995;33(5):1215–22.

Kilian M, Mikkelsen L, Henrichsen J. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int J Syst Bacteriol. 1989;39(4):471–84.

Killer J, Kopecny J, Mrazek J, Koppova I, Havlik J, Benada O, et al. *Bifidobacterium actinocoloniiforme* sp. nov. and *Bifidobacterium bohemicum* sp. nov., from the bumblebee digestive tract. Int J Syst Evol Microbiol. 2011;61(Pt 6):1315–21. doi:10.1099/ijs.0.022525-0.

Kilpper-Bälz R, Schleifer KH. Nucleic acid hybridization and cell wall composition studies of pyogenic streptococci. FEMS Microbiol Lett. 1984;24(2):355–64.

Kilpper-Bälz R, Schleifer K. Transfer of *Streptococcus morbillorum* to the genus Gemella as Gemella morbillorum comb. nov. Int J Syst Bacteriol. 1988;38(4):442–3.

Kim DS, Choi SH, Kim DW, Nam SH, Kim RN, Kang A et al. Genome Sequence of *Weissella cibaria* KACC 11862. J Bacteriol. 2010;193(3):797–8. doi:JB.01342-1010.1128.

Kim J, Chun J, Han H. *Leuconostoc kimchii* sp. nov., a new species from kimchi. Int J Syst Evol Microbiol. 2000;50(5):1915–9.

Kim B, Lee J, Jang J, Kim J, Han H. *Leuconostoc inhae* sp. nov., a lactic acid bacterium isolated from kimchi. Int J Syst Evol Microbiol. 2003;53(4):1123–6.

Kim MS, Roh SW, Bae JW. *Bifidobacterium stercoris* sp. nov., isolated from human faeces. Int J Syst Evol Microbiol. 2010;60(Pt 12):2823–7. doi:10.1099/ijs.0.019943-0.

King SJ, Leigh JA, Heath PJ, Luque I, Tarradas C, Dowson CG, et al. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis*: identification of virulent clones and potential capsular serotype exchange. J Clin Microbiol. 2002;40 (10):3671–80.

Klaenhammer T, Altermann E, Arigoni F, Bolotin A, Breidt F, Broadbent J, et al. Discovering lactic acid bacteria by genomics. Antonie Van Leeuwenhoek. 2002;82(1–4):29–58.

Klaenhammer TR, Barrangou R, Buck BL, Azcarate-Peril MA, Altermann E. Genomic features of lactic acid bacteria effecting bioprocessing and health. FEMS Microbiol Rev. 2005;29 (3):393–409. doi:10.1016/j.femsre.2005.04.007.

Klaenhammer TR, Altermann E, Pfeiler E, Buck BL, Goh YJ, O'Flaherty S, et al. Functional genomics of probiotic Lactobacilli. J Clin Gastroenterol. 2008;42(Suppl 3 Pt 2):S160–2. doi:10.1097/MCG.0b013e31817da140.

Klare I, Heier H, Claus H, Bohme G, Marin S, Seltmann G, et al. *Enterococcus faecium* strains with vanA-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. Microb Drug Resist. 1995;1(3):265–72.

Kleerebezem M, Hugenholtz J. Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol. 2003;14(2):232–7.

Klijn N, Weerkamp AH, de Vos WM. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. Appl Environ Microbiol. 1991;57(11):3390–3.

Klijn N, Weerkamp AH, De Vos W. Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystems. Appl Environ Microbiol. 1995;61(2):788–92.

Knudtson LM, Hartman PA. Routine procedures for isolation and identification of enterococci and fecal streptococci. Appl Environ Microbiol. 1992;58(9):3027–31.

Köhler G, Ludwig W, Heinz Schleifer K. Differentiation of lactococci by rRNA gene restriction analysis. FEMS Microbiol Lett. 1991;84(3):307–12.

Kojima M, Suda S, Hotta S, Hamada K, Suganuma A. Necessity of calcium ion for cell division in *Lactobacillus bifidus*. J Bacteriol. 1970;104(2):1010–3.

Koort J, Coenye T, Vandamme P, Sukura A, Björkroth J. *Enterococcus hermanniensis* sp. nov., from modified-atmosphere-packaged broiler meat and canine tonsils. Int J Syst Evol Microbiol. 2004;54(5):1823–7.

Kudo Y, Oki K, Watanabe K. *Lactobacillus delbrueckii* subsp. *sunkii* subsp. nov., isolated from sunki, a traditional Japanese pickle. Int J Syst Evol Microbiol. 2012;62(Pt 11):2643–9. doi:10.1099/ijs.0.037051-0.

Kullen MJ, Brady LJ, O'Sullivan DJ. Evaluation of using a short region of the *rec*A gene for rapid and sensitive speciation of dominant bifidobacteria in the human large intestine. FEMS Microbiol Lett. 1997;154(2):377–83.

Kurzak P, Ehrmann MA, Vogel RF. Diversity of lactic acid bacteria associated with ducks. Syst Appl Microbiol. 1998;21(4):588–92. doi:10.1016/S0723-2020(98)80071-4.

Kutahya OE, Starrenburg MJ, Rademaker JL, Klaassen CH, Vlieg JE, Smid EJ, et al. High-resolution amplified fragment length polymorphism typing of *Lactococcus lactis* strains enables identification of genetic markers for subspecies-related phenotypes. Appl Environ Microbiol. 2011;77(15):5192–8.

Ladner JT, Welch TJ, Whitehouse CA, Palacios GF. Genome sequence of *Weissella ceti* NC36, an emerging pathogen of farmed rainbow trout in the United States. Genome Announc. 2013;1 (1):e00187–e00212. doi:10.1128/genomeA.00187-12.

Lancefield R. A serological differentiation of human and other groups of hemolytic streptococci. J Exp Med. 1933;57:571–95.

Lauer E. *Bifidobacterium gallicum* sp. nov. isolated from human feces. Int J Syst Bacteriol. 1990;40(1):100–2.

Laukkanen-Ninios R, Didelot X, Jolley KA, Morelli G, Sangal V, Kristo P, et al. Population structure of the *Yersinia pseudotuberculosis* complex according to multilocus sequence typing. Environ Microbiol. 2011;13(12):3114–27.

Lawrence J, Yajko DM, Hadley WK. Incidence and characterization of beta-hemolytic *Streptococcus milleri* and differentiation from *S. pyogenes* (group A), *S. equisimilis* (group C), and large-colony group G streptococci. J Clin Microbiol. 1985;22(5):772–7.

Lazzi C, Bove CG, Sgarbi E, Monica G, La Gioia F, Sandra T et al. Application of AFLP fingerprint analysis for studying the biodiversity of *Streptococcus thermophilus*. J Microbiol Methods. 2009;79(1):48–54. doi:http://dx.doi.org/10.1016/j.mimet.2009.07.021.

le Dr ORLA-JENSENM. La classification des bactéries lactiques. Le Lait. 1924;4(36):468–74.

Lee Y. Characterization of *Weissella kimchii* PL9023 as a potential probiotic for women. FEMS Microbiol Lett. 2005;250(1):157–62. doi:10.1016/j.femsle.2005.07.009.

Lee JH, O'Sullivan DJ. Genomic insights into bifidobacteria. Microbiol Mol Biol Rev. 2010;74 (3):378–416. doi:10.1128/MMBR.00004-10.

Lee SH, Jung JY, Jeon CO. Complete genome sequence of *Weissella koreensis* KACC 15510, isolated from kimchi. J Bacteriol. 2011;193(19):5534. doi:193/19/5534.

Lee H, Park S, Kim J. Multiplex PCR-based detection and identification of *Leuconostoc* species. FEMS Microbiol Lett. 2000;193(2):243–7.

Lee JS, Lee KC, Ahn JS, Mheen TI, Pyun YR, Park YH. *Weissella koreensis* sp. nov., isolated from kimchi. Int J Syst Evol Microbiol. 2002;52(Pt 4):1257–61.

Lee JH, Bae JW, Chun J. Draft genome sequence of *Weissella koreensis* KCTC 3621T. J Bacteriol. 2012a;194(20):5711–2.

Lee SH, Park MS, Jung JY, Jeon CO. *Leuconostoc miyukkimchii* sp. nov., isolated from brown algae (Undaria pinnatifida) kimchi. Int J Syst Evol Microbiol. 2012b;62(5):1098–103.

Lefébure T, Stanhope MJ. Evolution of the core and pan-genome of Streptococcus: positive selection, recombination, and genome composition. Genome Biol. 2007;8(5):R71.

Lefevre J, Faucon G, Sicard A, Gasc A. DNA fingerprinting of *Streptococcus pneumoniae* strains by pulsed-field gel electrophoresis. J Clin Microbiol. 1993;31(10):2724–8.

Lemcke R, Bulte M. Occurrence of the vancomycin-resistant genes vanA, vanB, vanCl, vanC2 and vanC3 in *Enterococcus* strains isolated from poultry and pork. Int J Food Microbiol. 2000;60(2–3):185–94.

Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124(4):837–48. doi:10.1016/j.cell.2006.02.017.

Lindner P. Über ein neues in Malzmaischen vorkommendes, milchsäurebildendes Ferment. Wochenschrift fur Brauerei. 1887;4:437–40.

Lister J. A further contribution to the natural history of bacteria and the germ theory of fermentative changes. Quart J Microbiol Sci. 1873;13:380–408

Liu D, Wang C, Swiatlo EJ, Lawrence ML. PCR amplification of a species-specific putative transcriptional regulator gene reveals the identity of *Enterococcus faecalis*. Res Microbiol. 2005;156(9):944–8. doi:10.1016/j.resmic.2005.05.004.

Liu L, Zhang B, Tong H, Dong X. *Pediococcus ethanolidurans* sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar. Int J Syst Evol Microbiol. 2006;56(Pt 10):2405–8. doi:10.1099/ijs.0.64407-0.

Löhnis F. Die Benennung der Milchsaürebakterien. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. 1909;22:553–5.

Ludwig W, Seewaldt E, Kilpper-BÄ LZR, Heinz K, Magrum L, Woese CR, et al. The phylogenetic position of *Streptococcus* and *Enterococcus*. J Gen Microbiol. 1985;131(3):543–51.

Ludwig W, Weizenegger M, Kilpper-Bälz R, Schleifer K. Phylogenetic relationships of anaerobic streptococci. Int J Syst Bacteriol. 1988;38(1):15–8.

Maeda Y, Goldsmith C, Coulter W, Mason C, Dooley J, Lowery C, et al. Comparasion of five gene loci (rnpB, 16S rRNA, 16S-23S rRNA, sodA and dnaJ) to aid the molecular identification of viridans-group *streptococci* and *pneumococci*. Br J Biomed Sci. 2011;68(4):190.

Magnusson J, Jonsson H, Schnurer J, Roos S. *Weissella soli* sp. nov., a lactic acid bacterium isolated from soil. Int J Syst Evol Microbiol. 2002;52(Pt 3):831–4.

Maiden MC. Multilocus sequence typing of bacteria. Annu Rev Microbiol. 2006;60:561–88. doi:10.1146/annurev.micro.59.030804.121325.

Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 1998;95(6):3140–5.

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, et al. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A. 2006;103(42):15611–6. doi:10.1073/pnas.0607117103.

Makino H, Kushiro A, Ishikawa E, Muylaert D, Kubota H, Sakai T, et al. Transmission of intestinal *Bifidobacterium longum* subsp. longum strains from mother to infant, determined by multilocus sequencing typing and amplified fragment length polymorphism. Appl Environ Microbiol. 2011;77(19):6788–93. doi:10.1128/AEM.05346-11.

Manachini P, Flint S, Ward L, Kelly W, Fortina M, Parini C et al. Comparison between *streptococcus macedonicus* and *Streptococcus waius* strains and reclassification of *Streptococcus waius* (Flint et at. 1999) as *Streptococcus macedonicus* (Tsakalidou et al. 1998). Int J Syst Evol Microbiol. 2002;52(3):945–51.

Martin B, Garriga M, Hugas M, Aymerich T. Genetic diversity and safety aspects of enterococci from slightly fermented sausages. J Appl Microbiol. 2005;98(5):1177–90. doi:10.1111/j.1365-2672.2005.02555.x.

Martinez G, Harel J, Higgins R, Lacouture S, Daignault D, Gottschalk M. Characterization of *Streptococcus agalactiae* isolates of bovine and human origin by randomly amplified polymorphic DNA analysis. J Clin Microbiol. 2000;38(1):71–8.

Martinez-Murcia AJ, Collins MD. A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16 S rRNA. FEMS Microbiol Lett. 1990;58(1):73–83.

Martinez-Murcia AJ, Collins MD. A phylogenetic analysis of an atypical *Leuconostoc*: description of *Leuconostoc fallax* sp. nov. FEMS Microbiol Lett. 1991;66(1):55–9.

Martino ME, Maifreni M, Marino M, Bartolomeoli I, Carraro L, Fasolato L, et al. Genotypic and phenotypic diversity of *Pediococcus pentosaceus* strains isolated from food matrices and characterisation of the penocin operon. Antonie Van Leeuwenhoek. 2013;103(5):1149–63. doi:10.1007/s10482-013-9897-1.

Masco L, Ventura M, Zink R, Huys G, Swings J. Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. animalis subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov. Int J Syst Evol Microbiol. 2004;54(4):1137–43.

Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl Environ Microbiol. 1999;65(10):4506–12.

Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, et al. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol. 2002;68(11):5445–51.

Mees RH. Onderzoekingen over de Biersarcina. Thesis. Holland: Technical University Delft; 1934. p. 1–110.

Meslier V, Loux V, Renault P. Genome sequence of *Leuconostoc pseudomesenteroides* strain 4882, isolated from a dairy starter culture. J Bacteriol. 2012;194(23):6637.

Midha S, Ranjan M, Sharma V, Kumari A, Singh PK, Korpole S, et al. Genome sequence of *Pediococcus pentosaceus* strain IE-3. J Bacteriol. 2012;194(16):4468. doi:10.1128/JB.00897-12.

Milinovich GJ, Burrell PC, Pollitt CC, Bouvet A, Trott DJ. *Streptococcus henryi* sp. nov. and *Streptococcus caballi* sp. nov., isolated from the hindgut of horses with oligofructose-induced laminitis. Int J Syst Evol Microbiol. 2008;58(Pt 1):262–6. doi:10.1099/ijs.0.65063-0.

Miranda AG, Singh KV, Murray BE. Determination of the chromosomal size of three different strains of *Enterococcus faecalis* and one strain of *Enterococcus faecium*. DNA Cell Biol. 1992;11(4):331–5.

Miyake T, Watanabe K, Watanabe T, Oyaizu H. Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. Microbiol Immunol. 1998;42(10):661–7.

Mora D, Fortina MG, Parini C, Manachini PL. Identification of *Pediococcus acidilactici* and *Pediococcus pentosaceus* based on 16S rRNA and ldhD gene-targeted multiplex PCR analysis. FEMS Microbiol Lett. 1997;151(2):231–6.

Mora D, Parini C, Fortina MG, Manachini PL. Development of molecular RAPD marker for the identification of *Pediococcus acidilactici* strains. Syst Appl Microbiol. 2000;23(3):400–8. doi:10.1016/S0723-2020(00)80071-5.

Morita H, Nakano A, Onoda H, Toh H, Oshima K, Takami H, et al. *Bifidobacterium kashiwanohense* sp. nov., isolated from healthy infant faeces. Int J Syst Evol Microbiol. 2011a;61(Pt 11):2610–5. doi:10.1099/ijs.0.024521-0.

Morita H, Toh H, Oshima K, Yoshizaki M, Kawanishi M, Nakaya K, et al. Complete genome sequence and comparative analysis of the fish pathogen *Lactococcus garvieae*. PLoS One. 2011b;6(8):e23184.

Nadon C, Trees E, Ng L, Møller Nielsen E, Reimer A, Maxwell N. Development and application of MLVA methods as a tool for inter-laboratory surveillance. Euro Surveill. 2013;18:35.

Naimi A, Beck G, Branlant C. Primary and secondary structures of rRNA spacer regions in enterococci. Microbiology. 1997;143(Pt 3):823–34.

Nallapareddy SR, Duh RW, Singh KV, Murray BE. Molecular typing of selected *Enterococcus faecalis* isolates: pilot study using multilocus sequence typing and pulsed-field gel electrophoresis. J Clin Microbiol. 2002;40(3):868–76.

Naser S, Thompson FL, Hoste B, Gevers D, Vandemeulebroecke K, Cleenwerck I, et al. Phylogeny and identification of Enterococci by *atp*A gene sequence analysis. J Clin Microbiol. 2005;43(5):2224–30. doi:10.1128/JCM.43.5.2224-2230.2005.

Neish AS. Microbes in gastrointestinal health and disease. Gastroenterol. 2009;136(1):65–80. doi:10.1053/j.gastro.2008.10.080.

Nigatu A, Ahrne S, Gashe B, Molin G. Randomly amplified polymorphic DNA (RAPD) for discrimination of *Pediococcus pentosaceus* and *Ped. acidilactici* and rapid grouping of *Pediococcus* isolates. Lett Appl Microbiol. 1998;26(6):412–6.

Nocard M, Mollereau R. Sur une mammite contagieuse des vaches laitieres. Ann Inst Pasteur. 1887;1:109.

Nomura M, Kobayashi M, Narita T, Kimoto-Nira H, Okamoto T. Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. J Appl Microbiol. 2006;101 (2):396–405.

Ogier JC, Serror P. Safety assessment of dairy microorganisms: the *Enterococcus* genus. Int J Food Microbiol. 2008;126(3):291–301. doi:10.1016/j.ijfoodmicro.2007.08.017.

Ogier JC, Casalta E, Farrokh C, Saihi A. Safety assessment of dairy microorganisms: the *Leuconostoc* genus. Int J Food Microbiol. 2008;126(3):286–90.

Oh PL, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S, et al. Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. ISME J. 2010;4 (3):377–87. doi:10.1038/ismej.2009.123.

Oh SJ, Shin NR, Hyun DW, Kim PS, Kim JY, Kim MS, et al. *Weissella diestrammenae* sp. nov., isolated from the gut of a camel cricket (Diestrammena coreana). Int J Syst Evol Microbiol. 2013;63(Pt 8):2951–6. doi:10.1099/ijs.0.047548-0.

Okamoto M, Benno Y, Leung KP, Maeda N. *Bifidobacterium tsurumiense* sp. nov., from hamster dental plaque. Int J Syst Evol Microbiol. 2008;58(Pt 1):144–8. doi:10.1099/ijs.0.65296-0.

Ozawa Y, Courvalin P, Galimand M. Identification of enterococci at the species level by sequencing of the genes for D-alanine: D-alanine ligases. Syst Appl Microbiol. 2000;23 (2):230–7.

Padonou SW, Schillinger U, Nielsen DS, Franz CM, Hansen M, Hounhouigan JD, et al. *Weissella beninensis* sp. nov., a motile lactic acid bacterium from submerged cassava fermentations, and emended description of the genus *Weissella*. Int J Syst Evol Microbiol. 2009;60(Pt 9):2193–8. doi:10.1099/ijs.0.014332-0.

Park YJ, Oh EJ, Kim BK, Kim SM, Shim SI. Phenotypic characteristics of *Enterococcus faecium* variants confirmed by intergenic ribosomal polymerase chain reaction and *E. faecium* polymerase chain reaction. Diagn Microbiol Infect Dis. 1999;34(4):269–73.

Parker M. Streptococcus and Lactobacillus. In: Wilson GS, Miles AA, Parker MT, editors. Topley and Wilson's principles of bacteriology, virology and immunity. 7th ed. London: Edward Arnold; 1983. p. 173–217.

Parolo CC, Do T, Henssge U, Alves LS, de Santana Giongo FC, Corcao G et al. Genetic diversity of *Lactobacillus paracasei* isolated from in situ human oral biofilms. J Appl Microbiol. 2011;111(1):105–113.

Passerini D, Beltramo C, Coddeville M, Quentin Y, Ritzenthaler P, Daveran-Mingot M-L, et al. Genes but not genomes reveal bacterial domestication of *Lactococcus lactis*. PLoS One. 2010;5(12):e15306.

Patel R, Piper KE, Rouse MS, Steckelberg JM, Uhl JR, Kohner P, et al. Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. J Clin Microbiol. 1998;36(11):3399–407.

Pavel S, Devriese LA. Genus I. Enterococcus schleifer and Kilpper-Balz 1984. In: De-Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F et al., editors. Bergey's manual of systematic bacteriology. 2nd ed. Berlin: Springer; 2009. p. 594–607.

Pérez G, Cardell E, Zárate V. Random amplified polymorphic DNA analysis for differentiation of *Leuconostoc mesenteroides* subspecies isolated from Tenerife cheese. Lett Appl Microbiol. 2002;34(2):82–5.

Pérez T, Balcázar JL, Peix A, Valverde A, Velázquez E, de Blas I, et al. *Lactococcus lactis* subsp. *tructae* subsp. nov. isolated from the intestinal mucus of brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss). Int J Syst Evol Microbiol. 2011;61(8):1894–8.

Pfeiler EA, Klaenhammer TR. The genomics of lactic acid bacteria. Trends Microbiol. 2007;15 (12):546–53. doi:10.1016/j.tim.2007.09.010.

Picozzi C, Bonacina G, Vigentini I, Foschino R. Genetic diversity in Italian *Lactobacillus sanfranciscensis* strains assessed by multilocus sequence typing and pulsed-field gel electrophoresis analyses. Microbiology. 2010;156(Pt 7):2035–45.

Pittet V, Abegunde T, Marfleet T, Haakensen M, Morrow K, Jayaprakash T, et al. Complete genome sequence of the beer spoilage organism *Pediococcus claussenii* ATCC BAA-344T. J Bacteriol. 2012;194(5):1271–2. doi:10.1128/JB.06759-11.

Pot B, Ludwig W, Kersters K, Schleifer K-H. Taxonomy of lactic acid bacteria. In: Vuyst LD, Vandamme EJ, editors. Bacteriocins of lactic acid bacteria. London: Springer; 1994. p. 13–90.

Poupard JA, Husain I, Norris RF. Biology of the bifidobacteria. Bacteriol Rev. 1973;37(2):136–65.

Poyart C, Quesne G, Coulon S, Berche P, Trieu-Cuot P. Identification of streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase. J Clin Microbiol. 1998;36(1):41–7.

Poyart C, Quesne G, Trieu-Cuot P. Taxonomic dissection of the *Streptococcus bovis* group by analysis of manganese-dependent superoxide dismutase gene (*sod*A) sequences: reclassification of'*Streptococcus infantarius subsp. coli* as *Streptococcus lutetiensis* sp. nov. and of *Streptococcus bovis* biotype 11.2 as Streptococcus pasteurianus sp. nov. Int J Syst Evol Microbiol. 2002;52(4):1247–55.

Pridmore RD, Berger B, Desiere F, Vilanova D, Barretto C, Pittet AC, et al. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc Natl Acad Sci U S A. 2004;101(8):2512–7.

Rademaker JL, Herbet H, Starrenburg MJ, Naser SM, Gevers D, Kelly WJ, et al. Diversity analysis of dairy and nondairy *Lactococcus lactis* isolates, using a novel multilocus sequence analysis scheme and (GTG) 5-PCR fingerprinting. Appl Environ Microbiol. 2007;73 (22):7128–37.

Raftis EJ, Salvetti E, Torriani S, Felis GE, O'Toole PW. Genomic diversity of *Lactobacillus salivarius*. Appl Environ Microbiol. 2011;77(3):954–65.

Rahkila R, Johansson P, Sade E, Bjorkroth J. Identification of enterococci from broiler products and a broiler processing plant and description of *Enterococcus viikkiensis* sp. nov. Appl Environ Microbiol. 2011;77(4):1196–203. doi:10.1128/AEM.02412-10.

Ramachandran P, Lacher DW, Pfeiler EA, Elkins CA. Development of a tiered multilocus sequence typing scheme for members of the *Lactobacillus acidophilus* complex. Appl Environ Microbiol. 2013;79(23):7220–8. doi:10.1128/AEM.02257-13.

Ravelo C, Magariños B, López-Romalde S, Toranzo AE, Romalde JL. Molecular fingerprinting of fish-pathogenic *Lactococcus garvieae* strains by random amplified polymorphic DNA analysis. J Clin Microbiol. 2003;41(2):751–6.

Rodas AM, Ferrer S, Pardo I. 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. Syst Appl Microbiol. 2003;26(3):412–22.

Rosenbach FJ. Mikro-Organismen bei den. In: Bergman JF, editor. Wund-infections-krankheiten des menschen. Wiesbaden, Germany1884. p. 1–122.

Roy D, Sirois S. Molecular differentiation of Bifidobacterium species with amplified ribosomal DNA restriction analysis and alignment of short regions of the ldh gene. FEMS Microbiol Lett. 2000;191(1):17–24.

Roy D, Ward P, Champagne G. Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. Int J Food Microbiol. 1996;29(1):11–29.

Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C et al. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol. 2006;44 (6):2220–2228. doi:10.1128/JCM.02596-05.

Sabat AJ, Budimir A, Nashev D, Sa-Leao R, van Dijl J, Laurent F, et al. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill. 2013;18(4):20380.

Sakala RM, Hayashidani H, Kato Y, Hirata T, Makino Y, Fukushima A, et al. Change in the composition of the microflora on vacuum-packaged beef during chiller storage. Int J Food Microbiol. 2002a;74(1):87–99.

Sakala RM, Kato Y, Hayashidani H, Murakami M, Kaneuchi C, Ogawa M. *Lactobacillus fuchuensis* sp. nov., isolated from vacuum-packaged refrigerated beef. Int J Syst Evol Microbiol. 2002b;52(Pt 4):1151–4.

Salama M, Sandine W, Giovannoni S. Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. Appl Environ Microbiol. 1991;57 (5):1313–8.

Salvetti E, Torriani S, Felis GE. The genus Lactobacillus: a taxonomic update. Probiotics Antimicrob Proteins. 2012;4(4):217–26.

Salvetti E, Fondi M, Fani R, Torriani S, Felis GE. Evolution of lactic acid bacteria in the order Lactobacillales as depicted by analysis of glycolysis and pentose phosphate pathways. Syst Appl Microbiol. 2013;36(5):291–305. doi:10.1016/j.syapm.2013.03.009.

Samelis J, Rementzis J, Tsakalidou E, Metaxopoulos J. Usefulness of rapid GC analysis of cellular fatty acids for distinguishing *Weissella viridescens*, *Weissella paramesenteroides*, *Weissella hellenica* and some non-identifiable, arginine-negative Weissella strains of meat origin. Syst Appl Microbiol. 1998;21(2):260–5. doi:10.1016/S0723-2020(98)80031-3.

Satake S, Clark N, Rimland D, Nolte FS, Tenover FC. Detection of vancomycin-resistant enterococci in fecal samples by PCR. J Clin Microbiol. 1997;35(9):2325–30.

Saunders NA, Holmes A. Multilocus sequence typing (MLST) of *Staphylococcus aureus*. Methods Mol Biol. 2014;1085:113–30.

Scardovi V. The genus Bifidobacterium Orla-jensen 1924. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology. 8th ed. Baltimore: Williams & Wilkins Co.; 1986. p. 1418–34.

Scardovi V, Crociani F. *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, and *Bifidobacterium angulatum*: three new species and their deoxyribonucleic acid homology relationships. Int J Syst Bacteriol. 1974;24(1):6–20.

Scardovi V, Trovatelli LD. New species of bifidbacteria from Apis mellifica L. and Apis indica F. A contribution to the taxonomy and biochemistry of the genus Bifidobacterium. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. 1969;123(1):64–88.

Scardovi V, Trovatelli L, Biavati B, Zani G. *Bifidobacterium cuniculi*, *Bifidobacterium choerinum*, *Bifidobacterium boum*, and *Bifidobacterium pseudocatenulatum* four new species and their deoxyribonucleic acid homology relationships. Int J Syst Bacteriol. 1979;29(4):291–311.

Schillinger U, Boehringer B, Wallbaum S, Caroline L, Gonfa A, Huch Nee Kostinek M et al. A genus-specific PCR method for differentiation between Leuconostoc and Weissella and its application in identification of heterofermentative lactic acid bacteria from coffee fermentation. FEMS Microbiol Lett. 2008;286(2):222–6. doi:10.1111/j.1574-6968.2008.01286.x.

Schlegel L, Grimont F, Ageron E, Grimont PA, Bouvet A. Reappraisal of the taxonomy of the *Streptococcus bovis/Streptococcus equinus* complex and related species: description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *macedonicus* subsp. nov. and *S. gallolyticus* subsp. *pasteurianus* subsp. nov. Int J Syst Evol Microbiol. 2003;53(Pt 3):631–45.

Schleifer K. Recent changes in the taxonomy of lactic acid bacteria. FEMS Microbiol Lett. 1987;46(3):201–3.

Schleifer KH, Ehrmann M, Krusch U, Neve H. Revival of the species *Streptococcus thermophilus* (ex Orla-Jensen, 1919) nom. rev. Syst Appl Microbiol. 1991;14(4):386–8. doi:http://dx.doi.org/10.1016/S0723-2020(11)80314-0.

Schleifer KH, Kraus J, Dvorak C, Kilpper-Bälz R, Collins MD, Fischer W. Transfer of *Streptococcus lactis* and related Streptococci to the Genus Lactococcus gen. nov. Syst Appl Microbiol. 1985;6(2):183–95. doi:http://dx.doi.org/10.1016/S0723-2020(85)80052-7.

Schleifer K, Ludwig W. Phylogenetic relationships of lactic acid bacteria. In: Wood B, Holzapfel W, editors. The genera of lactic acid bacteria. London: Blackie Academic & Professional; 1995a. p. 7–18.

Schleifer KH, Ludwig W. Phylogeny of the genus Lactobacillus and related genera. Syst Appl Microbiol. 1995b;18(4):461–7.

Schleifer KHK-BR, Kraus J, Gehring F. Relatedness and classification of *Streptococcus mutans* and "mutans-like" streptococci. J Dent Res. 1984;63(8):1047–50.

Schottmüller H. Die Artunterscheidung der für den Menschen pathogenen Streptokokken durch Blutagar. Munchen Medical Wochenschr. 1903;50:908.

Seppälä H, He Q, Osterblad M, Huovinen P. Typing of group a streptococci by random amplified polymorphic DNA analysis. J Clin Microbiol. 1994;32(8):1945–8.

Sgorbati B, Biavati B, Palenzona D. The genus Bifidobacterium. In: Wood B, Holzapfel W, editors. The genera of lactic acid bacteria. London: Blackie Academic & Professional; 1995. p. 279–306.

Sharpe ME, Garvie EI, Tilbury RH. Some slime-forming heterofermentative species of the genus Lactobacillus. Appl Microbiol. 1972;23(2):389–97.

Shaw BG, Harding CD. *Leuconostoc gelidum* sp. nov. and *Leuconostoc carnosum* sp. nov. from chill-stored meats. Soc General Microbiol. 1989;39(3):217–23.

Sherman JM. The streptococci. Bacteriol Rev. 1937;1(1):3–97.

Siezen RJ, Bayjanov J, Renckens B, Wels M, van Hijum SA, Molenaar D, et al. Complete genome sequence of *Lactococcus lactis* subsp. *lactis* KF147, a plant-associated lactic acid bacterium. J Bacteriol. 2010;192(10):2649–50.

Simpson W, Taguchi H. The genus Pediococcus, with notes on the genera Tetratogenococcus and Aerococcus. In: Wood B, Holzapfel W, editors. The genera of lactic acid bacteria. London: Blackie Academic & Professional; 1995. p. 125–72.

Simpson PJ, Stanton C, Fitzgerald GF, Ross RP. Genomic diversity within the genus Pediococcus as revealed by randomly amplified polymorphic DNA PCR and pulsed-field gel electrophoresis. Appl Environ Microbiol. 2002;68(2):765–71.

Skerman VBD, McGowan V, Sneath PHA. Approved lists of bacterial names, vol 1. Baltimore: Williams & Wilkins; 1980.

Smit G, Smit BA, Engels WJ. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol Rev. 2005;29(3):591–610.

Snauwaert I, Papalexandratou Z, De Vuyst L, Vandamme P. Characterization of strains of *Weissella fabalis* sp. nov. and Fructobacillus tropaeoli from spontaneous cocoa bean fermentations. Int J Syst Evol Microbiol. 2012;63(Pt 5):1709–16. doi:10.1099/ijs.0.040311-0.

Stackebrandt E, Goebel B. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol. 1994;44(4):846–9.

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PA, Kampfer P, Maiden MC, et al. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol. 2002;52(Pt 3):1043–7.

Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol. 1997;36(1):1–29.

Suerbaum S, Lohrengel M, Sonnevend A, Ruberg F, Kist M. Allelic diversity and recombination in Campylobacter jejuni. J Bacteriol. 2001;183(8):2259–553.

Svec P, Vancanneyt M, Seman M, Snauwaert C, Lefebvre K, Sedlacek I, et al. Evaluation of (GTG)5-PCR for identification of *Enterococcus* spp. FEMS Microbiol Lett. 2005;247(1):59–63. doi:10.1016/j.femsle.2005.04.030.

Tailliez P, Tremblay J, Ehrlich SD, Chopin A. Molecular diversity and relationship within *Lactococcus lactis*, as revealed by randomly amplified polymorphic DNA (RAPD). Syst Appl Microbiol. 1998;21(4):530–8. doi:http://dx.doi.org/10.1016/S0723-2020(98)80065-9.

Takada K, Hayashi K, Sato Y, Hirasawa M. *Streptococcus dentapri* sp. nov., isolated from the wild boar oral cavity. Int J Syst Evol Microbiol. 2010;60(Pt 4):820–3. doi:10.1099/ijs.0.012799-0.

Takada K, Saito M, Tsudukibashi O, Hiroi T, Hirasawa M. *Streptococcus orisasini* sp. nov. and *Streptococcus dentasini* sp. nov., isolated from the oral cavity of donkeys. Int J Syst Evol Microbiol. 2013;63(Pt 8):2782–6. doi:10.1099/ijs.0.047142-0.

Tanasupawat S, Shida O, Okada S, Komagata K. *Lactobacillus acidipiscis* sp. nov. and *Weissella thailandensis* sp. nov., isolated from fermented fish in Thailand. Int J Syst Evol Microbiol. 2000;50(Pt 4):1479–85.

Tanasupawat S, Pakdeeto A, Thawai C, Yukphan P, Okada S. Identification of lactic acid bacteria from fermented tea leaves (miang) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov. J Gen Appl Microbiol. 2007;53(1):7–15.

Tanganurat W, Quinquis B, Leelawatcharamas V, Bolotin A. Genotypic and phenotypic characterization of *Lactobacillus plantarum* strains isolated from Thai fermented fruits and vegetables. J Basic Microbiol. 2009;49(4):377–85.

Tanigawa K, Watanabe K. Multilocus sequence typing reveals a novel subspeciation of *Lactobacillus delbrueckii*. Microbiology. 2011;157(Pt 3):727–38.

Tanigawa K, Kawabata H, Watanabe K. Identification and typing of *Lactococcus lactis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol. 2010;76(12):4055–62.

Tanskanen EI, Tulloch DL, Hillier AJ, Davidson BE. Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. Appl Environ Microbiol. 1990;56(10):3105–11.

Täpp J, Thollesson M, Herrmann B. Phylogenetic relationships and genotyping of the genus Streptococcus by sequence determination of the RNase P RNA gene, *rnp*B. Int J Syst Evol Microbiol. 2003;53(6):1861–71.

Tardif G, Sulavik M, Jones G, Clewell D. Spontaneous switching of the sucrose-promoted colony phenotype in *Streptococcus sanguis*. Infect Immun. 1989;57(12):3945–8.

Tettelin H, Masignani V, Cieslewicz MJ, Eisen JA, Peterson S, Wessels MR, et al. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. Proc Natl Acad Sci U S A. 2002;99(19):12391–6.

Thiercelin E, Jouhaud L. Reproduction de l'enterocoque; taches centrales; granulations peripheriques et microblastes. Comptes Rendus des Seances de la Societe de Biologie Paris. 1903;55:686–8.

Tissier H. Le bacterium coli et la reaction chromophile d'Escherich. Crit Rev Soc Biol. 1899;51:943–5.

Tissier H. Recherches sur la flore intestinale des nourrissons (etat normal et pathologique). M.D. thesis. Paris, France: University of Paris; 1900.

Titze-de-Almeida R, Willems RJ, Top J, Rodrigues IP, Ferreira RF, Boelens H, et al. Multilocus variable-number tandem-repeat polymorphism among Brazilian *Enterococcus faecalis* strains. J Clin Microbiol. 2004;42(10):4879–81.

Titze-de-Almeida R, Van Belkum A, Felipe MS, Zanella RC, Top J, Willems RJ. Multilocus sequence typing of hospital-associated *Enterococcus faecium* from Brazil reveals their unique evolutionary history. Microb Drug Resist. 2006;12(2):121. doi:10.1089/mdr.2006.12.121.

Todd E, Hewitt L. A new culture medium for the production of antigenic streptococcal haemolysin. J Pathol Bacteriol. 1932;35(6):973–4.

Tohno M, Kitahara M, Inoue H, Uegaki R, Irisawa T, Ohkuma M, et al. *Weissella oryzae* sp. nov., isolated from fermented rice grains. Int J Syst Evol Microbiol. 2012;63(Pt 4):1417–20. doi:10.1099/ijs.0.043612-0.

Top J, Schouls LM, Bonten MJ, Willems RJ. Multiple-locus variable-number tandem repeat analysis, a novel typing scheme to study the genetic relatedness and epidemiology of *Enterococcus faecium* isolates. J Clin Microbiol. 2004;42(10):4503–11. doi:10.1128/JCM.42.10.4503-4511.2004.

Torriani S, Felis GE, Dellaglio F. Differentiation of *Lactobacillus plantarum, L. pentosus*, and *L. paraplantarum* by *rec*A gene sequence analysis and multiplex PCR assay with *rec*A gene-derived primers. Appl Environ Microbiol. 2001;67(8):3450–4.

Truong T, Menard C, Mouton C, Trahan L. Identification of mutans and other oral streptococci by random amplified polymorphic DNA analysis. J Med Microbiol. 2000;49(1):63–71.

Ulrich A, Müller T. Heterogeneity of plant-associated streptococci as characterized by phenotypic features and restriction analysis of PCR-amplified 16S rDNA. J Appl Microbiol. 1998;84(2):293–303.

Urbach E, Daniels B, Salama MS, Sandine WE, Giovannoni SJ. The ldh phylogeny for environmental isolates of *Lactococcus lactis* is consistent with rRNA genotypes but not with phenotypes. Appl Environ Microbiol. 1997;63(2):694–702.

van den Braak N, van Belkum A, van Keulen M, Vliegenthart J, Verbrugh HA, Endtz HP. Molecular characterization of vancomycin-resistant enterococci from hospitalized patients and poultry products in The Netherlands. J Clin Microbiol. 1998;36(7):1927–32.

Vancanneyt M, Lombardi A, Andrighetto C, Knijff E, Torriani S, Björkroth KJ, et al. Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. Appl Environ Microbiol. 2002;68(3):1381–91.

Vancanneyt M, Zamfir M, De Wachter M, Cleenwerck I, Hoste B, Rossi F, et al. Reclassification of *Leuconostoc argentinum* as a later synonym of *Leuconostoc lactis*. Soc General Microbiol. 2006;56(1):213–6.

Vandamme P, Pot B, Falsen E, Kersters K, Devriese LA. Taxonomic study of Lancefield streptococcal groups C, G, and L (*Streptococcus dysgalactiae*) and proposal of *S. dysgalactiae* subsp. equisimilis subsp. nov. Int J Syst Bacteriol. 1996a;46(3):774–81.

Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev. 1996b;60(2):407–38.

Vela AI, Fernandez A, de Quiros YB, Herraez P, Dominguez L, Fernandez-Garayzabal JF. *Weissella ceti* sp. nov., isolated from beaked whales (Mesoplodon bidens). Int J Syst Evol Microbiol. 2011;61(Pt 11):2758–62. doi:10.1099/ijs.0.028522-0.

Venema K, Maathuis AJ. A PCR-based method for identification of bifidobacteria from the human alimentary tract at the species level. FEMS Microbiol Lett. 2003;224(1):143–9.

Ventura M, van Sinderen D, Fitzgerald GF, Zink R. Insights into the taxonomy, genetics and physiology of bifidobacteria. Antonie Van Leeuwenhoek. 2004;86(3):205–23. doi:10.1023/B:ANTO.0000047930.11029.ec.

Ventura M, Zink R. Rapid identification, differentiation, and proposed new taxonomic classification of *Bifidobacterium lactis*. Appl Environ Microbiol. 2002;68(12):6429–34.

Ventura M, Zink R. Comparative sequence analysis of the tuf and recA genes and restriction fragment length polymorphism of the internal transcribed spacer region sequences supply additional tools for discriminating *Bifidobacterium lactis* from *Bifidobacterium animalis*. Appl Environ Microbiol. 2003;69(12):7517–22.

Ventura M, Elli M, Reniero R, Zink R. Molecular microbial analysis of Bifidobacterium isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). FEMS Microbiol Ecol. 2001a;36(2–3):113–21.

Ventura M, Reniero R, Zink R. Specific identification and targeted characterization of *Bifidobacterium lactis* from different environmental isolates by a combined multiplex-PCR approach. Appl Environ Microbiol. 2001b;67(6):2760–5. doi:10.1128/AEM.67.6.2760-2765.2001.

Ventura M, Canchaya C, Meylan V, Klaenhammer TR, Zink R. Analysis, characterization, and loci of the *tuf* genes in lactobacillus and bifidobacterium species and their direct application for species identification. Appl Environ Microbiol. 2003a;69(11):6908–22.

Ventura M, Meylan V, Zink R. Identification and tracing of Bifidobacterium species by use of enterobacterial repetitive intergenic consensus sequences. Appl Environ Microbiol. 2003b;69(7):4296–301.

Ventura M, Canchaya C, van Sinderen D, Fitzgerald GF, Zink R. *Bifidobacterium lactis* DSM 10140: identification of the atp (atpBEFHAGDC) operon and analysis of its genetic structure, characteristics, and phylogeny. Appl Environ Microbiol. 2004a;70(5):3110–21.

Ventura M, Canchaya C, Zink R, Fitzgerald GF, van Sinderen D. Characterization of the groEL and groES loci in *Bifidobacterium breve* UCC 2003: genetic, transcriptional, and phylogenetic analyses. Appl Environ Microbiol. 2004b;70(10):6197–209. doi:10.1128/AEM.70.10.6197-6209.2004.

Ventura M, Zink R, Fitzgerald GF, van Sinderen D. Gene structure and transcriptional organization of the *dna*K operon of *Bifidobacterium breve* UCC 2003 and application of the operon in bifidobacterial tracing. Appl Environ Microbiol. 2005;71(1):487–500. doi:10.1128/AEM.71.1.487-500.2005.

Ventura M, Canchaya C, Del Casale A, Dellaglio F, Neviani E, Fitzgerald GF, et al. Analysis of bifidobacterial evolution using a multilocus approach. Int J Syst Evol Microbiol. 2006;56(Pt 12):2783–92. doi:10.1099/ijs.0.64233-0.

Villani F, Moschetti G, Blaiotta G, Coppola S. Characterization of strains of *Leuconostoc mesenteroides* by analysis of soluble whole-cell protein pattern, DNA fingerprinting and restriction of ribosomal DNA. J Appl Microbiol. 1997;82(5):578–88.

Vincent D, Roy D, Mondou F, Dery C. Characterization of bifidobacteria by random DNA amplification. Int J Food Microbiol. 1998;43(3):185–93.

Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol. 2001;67(6):2578–85. doi:10.1128/AEM.67.6.2578-2585.2001.

Wang LT, Kuo HP, Wu YC, Tai CJ, Lee FL. *Lactobacillus taiwanensis* sp. nov., isolated from silage. Int J Syst Evol Microbiol. 2009;59(8):2064–8.

Watanabe K, Fujimoto J, Tomii Y, Sasamoto M, Makino H, Kudo Y, et al. *Lactobacillus kisonensis* sp. nov., *Lactobacillus otakiensis* sp. nov., *Lactobacillus rapi* sp. nov. and *Lactobacillus sunkii* sp. nov., heterofermentative species isolated from sunki, a traditional Japanese pickle. Int J Syst Evol Microbiol. 2009;59(Pt 4):754–60. doi:10.1099/ijs.0.004689-0.

Wegmann U, O'Connell-Motherway M, Zomer A, Buist G, Shearman C, Canchaya C, et al. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. cremoris MG1363. J Bacteriol. 2007;189(8):3256–70.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991;173(2):697–703.

Werner G, Klare I, Witte W. The current MLVA typing scheme for *Enterococcus faecium* is less discriminatory than MLST and PFGE for epidemic-virulent, hospital-adapted clonal types. BMC Microbiol. 2007;7:28. doi:10.1186/1471-2180-7-28.

Whatmore AM, Whiley RA. Re-evaluation of the taxonomic position of *Streptococcus ferus*. Int J Syst Evol Microbiol. 2002;52(5):1783–7.

Whiley RA, Beighton D. Emended descriptions and recognition of *Streptococcus constellatus*, *Streptococcus intermedius*, and *Streptococcus anginosus* as distinct species. Int J Syst Bacteriol. 1991;41(1):1–5.

Whiley R, Hall L, Hardie J, Beighton D. A study of small-colony, β-haemolytic, Lancefield group C streptococci within the anginosus group: description of *Streptococcus constellatus* subsp. *pharyngis* subsp. nov., associated with the human throat and pharyngitis. Int J Syst Evol Microbiol. 1999;49(4):1443–9.

Wijtzes T, Bruggeman M, Nout M, Zwietering M. A computerised system for the identification of lactic acid bacteria. Int J Food Microbiol. 1997;38(1):65–70.

Willems RJ, Top J, van Den Braak N, van Belkum A, Endtz H, Mevius D et al. Host specificity of vancomycin-resistant *Enterococcus faecium*. J Infect Dis. 2000;182(3):816–23. doi:10.1086/315752.

Willems RJ, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, et al. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerg Infect Dis. 2005;11(6):821–8. doi:10.3201/eid1106.041204.

Williams AM, Fryer JL, Collins MD. *Lactococcus piscium* sp. nov. a new *Lactococcus* species from salmonid fish. FEMS Microbiol Lett. 1990;68(1–2):109–13. doi:http://dx.doi.org/10.1016/0378-1097(90)90134-C.

Williams AM, Collins M. Molecular taxonomic studies on *Streptococcus uberis* types I and II. description of *Streptococcus parauberis* sp. nov. J Appl Microbiol. 1990;68(5):485–90.

Woese CR. Bacterial evolution. Microbiol Rev. 1987;51(2):221–71.

Xu HY, Sun ZH, Liu WJ, Yu J, Song YQ, Lv Q et al. Multilocus sequence typing of *Lactococcus Lactis* from naturally fermented milk foods in Ethnic Minority Areas of China. J Dairy Sci. 2013;in press.

Xu P, Alves JM, Kitten T, Brown A, Chen Z, Ozaki LS, et al. Genome of the opportunistic pathogen *Streptococcus sanguinis*. J Bacteriol. 2007;189(8):3166–75. doi:10.1128/JB.01808-06.

Yaeshima T, Fujisawa T, Mitsuoka T. *Bifidobacterium globosum*, subjective synonym of *Bifidobacterium pseudolongum*, and description of *Bifidobacterium pseudolongum* subsp. *pseudolongum* comb. nov. and *Bifidobacterium pseudolongum* subsp. *globosum* comb. nov. Syst Appl Microbiol. 1992;15(3):380–5.

Yost CK, Nattress FM. The use of multiplex PCR reactions to characterize populations of lactic acid bacteria associated with meat spoilag. Lett Appl Microbiol. 2000;31(2):129–33.

Zhang B, Tong H, Dong X. *Pediococcus cellicola* sp. nov., a novel lactic acid coccus isolated from a distilled-spirit-fermenting cellar. Int J Syst Evol Microbiol. 2005;55(Pt 5):2167–70. doi:10.1099/ijs.0.63778-0.

Zhang ZG, Ye ZQ, Yu L, Shi P. Phylogenomic reconstruction of lactic acid bacteria: an update. BMC Evol Biol. 2011;11:1. doi:10.1186/1471-2148-11-1.

Zhang M, Yan L, Zhu G, Holifield M, Todd D, Zhang S. *Streptococcus troglodytidis* sp. nov., isolated from a foot abscess of a chimpanzee (Pan troglodytes). Int J Syst Evol Microbiol. 2013;63(Pt 2):449–53. doi:10.1099/ijs.0.038133-0.

Zhu H, Willcox M, Knox K. A new species of oral *Streptococcus* isolated from Sprague-Dawley rats, *Streptococcus orisratti* sp. nov. Int J Syst Evol Microbiol. 2000;50(1):55–61.

Zilber-Rosenberg I, Rosenberg E. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol Rev. 2008;32(5):723–35. doi:10.1111/j.1574-6976.2008.00123.x.

Zou Y, Liu F, Fang C, Wan D, Yang R, Su Q, et al. *Lactobacillus shenzhenensis* sp. nov., isolated from a fermented dairy beverage. Int J Syst Evol Microbiol. 2013;63(Pt 5):1817–23. doi:10.1099/ijs.0.041111-0.

Chapter 2
Biodiversity of Lactic Acid Bacteria

Wenjun Liu, Huili Pang, Heping Zhang and Yimin Cai

Abstract Lactic acid bacteria (LAB) are regarded the most important bacteria concerning food fermentation, pharmaceutical and special dietary applications. The most commonly used strains of different LAB species in food including the genera of *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Bifidobacterium* are updated and described on taxonomy and description based on physiological and biochemical characteristics. Diversity of LAB in different traditional fermented foods (especially dairy products, fermented vegetable food and meat-based food) is reviewed in this chapter. The LAB variable component and predominated species in different foods and the same food products in different places is introduced briefly. Specifically, the biodiversity of lactic acid bacteria in silage is reviewed at the end of this chapter.

Keywords Lactic acid bacteria · Diversity · Fermented food · Silage

W. Liu (✉) · H. Zhang
Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education,
Inner Mongolia Agricultural University, Hohhot, China
e-mail: wjliu168@163.com

H. Zhang
e-mail: hepingdd@vip.sina.com

H. Pang
Zhengzhou University, Zhengzhou, People's Republic of China
e-mail: pang@zzu.edu.cn

Y. Cai
Crop, Livestock and Environment Division, Japan International Research Centre
for Agricultural Sciences, 1-1 Ohwashi, Ibaragi, Tsukuba 305-8686, Japan
e-mail: cai@affrc.go.jp

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_2,
© Springer Science+Business Media Dordrecht 2014

2.1 Introduction

2.1.1 General Background

The concept of lactic acid bacteria (LAB) as a group of organisms developed at the beginning of the 1900s. The first pure culture ('*Bacterium lactis,* now known as *Lactococcus lactis'*) was isolated in 1873 by Lister (Fennema et al. 2004). LAB are historically defined as a ubiquitous and heterogeneous family of microbes that can ferment a variety of nutrients into, primarily, lactic acid although recent molecular evidence has challenged this definition (Brooijmans et al. 2009). They are found in environments rich in available carbohydrate substrates, such as food and feed, but also in human and animal cavities, and in sewage and plant material. Indeed, strains have been isolated from all these environments (Kandler and Weiss 1986). Besides lactic acid, other side products include acetate, ethanol, CO_2, formate and succinate (Hammes and Vogel 1995; Hammes and Hertel 2009).

The general characteristics of LAB are that they are gram-positive, catalase negative (although some strains can produce pseudocatalase), anaerobic or microaerophilic, acid-tolerant and non-sporulating rods and cocci (Orla-Jensen 1919). Traditionally, LAB were called 'milk–souring organisms', and often negatively associated with loss of food and feed due to fermentation. However, LAB are increasingly considered as beneficial microorganisms; some strains are even thought to be health promoting (probiotic). Of all the bacteria exploited for domestic use, the LAB are the most widely studied and are exploited in numerous industrial applications ranging from starter cultures in the dairy industry to probiotics in dietary (Konigs et al. 2000) supplements and bio-conversion agents (Adams 1999).

LAB are found in two distinct phyla, namely *Firmicutes* and *Actinobacteria*. Within the *Firmicutes*, LAB belong to the order *Lactobacillales* and include the following genera: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Symbiobacterium*, *Tetragenococcus*, *Vagococcus* and *Weissella*, which are all low guanine–cytosine content organisms (31–49 %) (Horvath et al. 2009). LAB in *Actinobacteria* phylum only includes species of *Bifidobacterium* genus.

LAB obtain their energy by substrate-level phosphorylation because they do not posses a functional respiratory system. Two basic hexose fermentative pathways are used. The homofermentative pathway is based on the Embden–Meyerhof–Parnas pathway and produces virtually only lactic acid as the end product. The heterofermentative or heterolactic pathway (also known as the pentose phosphoketolase pathway, the hexose monophosphate shunt, or the 6-phosphogluconate pathway) produces not only lactic acid as the end product, but also significant amounts of CO_2 and ethanol or acetate (Kandler 1983; Lahtinen et al. 2011). Historically, the mode of fermentation in combination with physiological characteristics such as temperature ranges for growth and sugar utilisation patterns

were used as classification criteria to allocate LAB to genera and species. With the application of modern taxonomic tools (especially molecular methods) to LAB identification and classification some flaws in taxonomy based on physiology have been identified although these attributes still remain very important for the classification and ultimate exploitation of LAB.

2.1.2 Sources of LAB

LAB are found in diverse habitats wherever carbohydrate substrates are available. These include food and feed (dairy products, grain products, meat and fish products, beer, wine, fruits and fruit juices, pickled vegetables, mash, sauerkraut, silage and sourdough), water, soil, sewage and the oral (mucous membranes), respiratory, gastrointestinal and genital tracts of humans and animals (Horvath et al. 2009; Klaenhammer et al. 2002, 2005; Kleerebezem and Hugenholtz 2003).

The largest genus, *Lactobacillus*, includes over 100 species that are ubiquitous wherever substances rich in carbohydrates are available. They are closely associated with terrestrial and marine animals, their environment (plants, materials of plant origin, manure) and their food (cheese, yogurt) and most commonly found in the body cavities of humans and animals (Ring and Gatesoupe 1998; Tailliez 2001). In humans they are present in the oral cavity (1×10^3 to 1×10^7 colony forming units (CFU)/g, the ileum (1×10^3 to 1×10^7 CFU/g) and the colon (1×10^4 to 1×10^8 CFU/g) and are the dominant microorganism in the vagina (Hill et al. 1984; Forsum et al. 2005; Merk et al. 2005). *Lactobacillus* spp. have been found in the gastrointestinal tract of humans, pigs, chickens, cattle, dogs, mice, rats and hamsters (Hammes and Hertel 2009).

To date, 154 species from the genus *Lactobacillus* have been validly published isolated from different sources (http://www.bacterio.net/lactobacillus.html). Here we classify these sources into 11 major types (Fig. 2.1). The majority of species (almost a third of those described) were isolated from human and animal intestinal tracts and faeces (shown in Fig. 2.1). Vegetables and their associated fermentation products (including pickle and kimchi, sourdough etc.) provided the second largest number of *Lactobacillus* species isolated.

Species from other genera such as *Enterococcus* and *Bifidobacterium* were also mainly isolated from the intestinal tracts, faeces and skin of animals or humans (Felis and Dellaglio 2007). Species from the genus *Leuconostoc* were mainly isolated from chill-stored meats or clinical sources, although they were also found in association with plant material, fermented dairy products and wines (Thunell 1995). Species from the genus *Pediococcus* have long been associated with spoilage of beer as they produce diacetyl during fermentation and are most often isolated from spoiled beer, distilled material, or from cellars used for fermenting (Stiles and Holzapfel 1997). Other *Pediococcus* species, particularly *Pediococcus pentosaceus*, have also been isolated from dairy products (Tzanetakis and Litopoulou-Tzanetaki 1989).

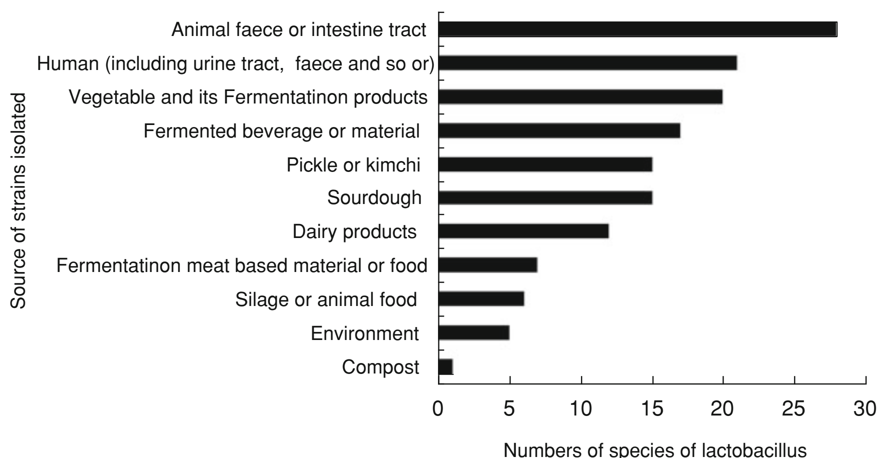


Fig. 2.1 Original source of the type specimens of *Lactobacillus* species that have been isolated

Species from the genus *Lactococcus* have been isolated from plant material (Cai et al. 2011; Chen et al. 2012), but are most commonly associated with dairy products (Teuber 1995).

2.2 Diversity in LAB

Early in the twentieth century, the term 'Lactic acid bacteria', or LAB, was first coined in the monograph of Orla-Jensen (1919) and this still forms the basis of our current classification system. The criteria for description of bacteria used by Orla-Jensen (1919) include cellular morphology, mode of glucose fermentation, temperature ranges for growth and sugar utilisation patterns (Table 2.1). According to the current taxonomic classification LAB belong to the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales* (Lahtinen et al. 2011); the different families include *Aerococcaceae*, *Carnobacteriacea*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Streptococcaceae* (http://www.uniprot.org/taxonomy/186826). The genera of LAB that are associated with food are *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Tetragenococcus*, *Vagococcus*, *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Weissella*, *Lactococcus* and *Streptococcus*. Phylogenetically, all the above-mentioned genera form a clade belonging to the clostridial branch of Gram-positive bacteria with low guanine–cystine content (<50 %). This property distanced these 'traditional' LABs from the bifidobacteria which have greater than 55 % guanine–cystine content and belong to the 'Actinomyces' branch of bacteria (Schleifer and Ludwig 1995a, b). Nevertheless, the genus *Bifidobacterium* is still regarded as a LAB by some researchers, because of its similar physiological and biochemical properties and because it shares some common ecological niches such as the gastrointestinal tract (Klein et al. 1998).

Table 2.1 Genera of LAB associated with food and their physiological characteristics

Family	Genera	Characteristics								
		Shape	CO_2 from glucose	Growth at 10 °C	Growth at 45 °C	Growth in 6.5 % NaCl	Growth in 18 % NaCl	Growth at pH 4.4	Growth at pH 9.6	Type of lactic acid
Aerococcaceae	*Aerococcus*	Cocci (tetrads)	–	+	–	+	–	–	+	L
Carnobacteriaceae	*Carnobacterium*	Rods	–	+	–	ND	–	ND	–	L
Enterococcaceae	*Enterococcus*	Cocci	–	+	+	+	–	+	–	L
	Tetrageonococcus	Cocci (tetrads)		+	–	+	+	Variable	+	L
	Vagococcus	Cocci		+	–	–	–	ND	–	ND
	Pediococcus	Cocci (tetrads)	–	Variable	Variable	Variable	–	+	–	D, L, DL
Lactobacillaceae	*Lactobacillus*	Rods	Variable	Variable	Variable	Variable	–	ND	L	D, L, DL
Leuconostocaecae	*Leuconostoc*	Cocci	+	+	–	Variable	–	Variable	–	D
	Oenococcus	Cocci	+	+	–	Variable	–	Variable	–	D
	Weissella	Cocci[a]	+	+	–	Variable	–	Variable	–	D, DL
Streptococcaceae	*Lactococcus*[b]	Cocci	–	+	–	–	–	Variable	–	L
	Streptococcus	Cocci	–	–	Variable	–	–	–	–	L

The table is compiled from the work of Lahtinen et al. (2011) and Fennema et al.(2004)

Note ND Not Determined

[a] Some *Weissella* strains are rod shaped

[b] In the old literature, *Lactococcus* species are referred to as Group *N Streptococci*

2.2.1 *Description and Taxonomy of LAB Genera*

2.2.1.1 The Genus *Aerococcus*

The genus *Aerococcus* was created by Williams et al. (1953) to accommodate some species that were Gram-positive, microaerophilic and catalase negative (although some display weak nonheme pseudocatalase activity); these coccoid organisms differed from *Streptococcus* species primarily by their characteristic tetrad cellular arrangement. *Aerococcus* species have been found from a wide range of environments including air, dust, vegetation, meat-curing brines, soil, human respiratory tracts and marine sources. For a long time, the genus contained only a single species, *Aerococcus viridans* and historically, this species was thought to resemble *Pediococcus* species more than *Streptococcus* species on account of the cellular morphology. However, more recently, six other *Aerococcus* species have been described from human sources: *Aerococcus christensenii, Aerococcus sanguinicola, Aerococcus suis*, *Aerococcus urinae*, *Aerococcus urinaehominis* and *Aerococcus viridans* (Garvie 1988; Aguirre and Collins 1992; Collins et al. 1999; Lawson et al. 2001a, b; Felis et al. 1934). Comparative 16S rRNA gene sequences studies showed that this expanded *Aerococcus* genus formed a robust group among the catalase negative, Gram-positive cocci (Collins et al. 1999; Lawson et al. 2001a, b). Phylogenetically, *Aerococcus* is distinct from other genera such as *Streptococcus* and *Pediococcus*. A phylogenetic tree depicting the interrelationships between *Aerococcus* spp. and their close relatives can be found in Bergey's Manual of Systematic Bacteriology (Hammes and Hertel 2009).

Although the genus *Aerococcus* is phylogenetically distinct, using phenotypic traits, it is difficult to distinguish it from related genera that have a coccoid shape. In contrast, the species within the genus *Aerococcus* are very easily distinguished from each other using conventional and miniaturised API test systems (Hammes and Hertel 2009) (Table 2.2).

2.2.1.2 The Genus *Carnobacterium*

The genus *Carnobacterium* was first proposed by Collins et al. (1987) to accommodate the group of 'atypical lactobacilli' strains isolated from vacuum-packed meat that are also unable to grow on acetate agar (Collins et al. 1987). This description included two group strains that were rod shaped, Gram-positive, catalase negative and non-spore forming that had been isolated from poultry meat stored at low temperatures (Thornley 1957) and vacuum packed, chilled stored meat (Shaw and Harding 1984). The strains in these groups had previously been referred to as 'non-aciduric *Lactobacillus*'. Previously, two groups of non-aciduric *Lactobacillus* had also been described and proposed as being new species, specifically *Lb. divergens* (Holzapfel and Gerber 1983) and *Lb. carnis*

Table 2.2 Conventional and API system tests for distinguishing between species in the genus *Aerococcus*

Characteristics		*A. viridans*	*A. christensenii*	*A. sanguinicola*	*A. urinae*	*A. urinaeequi*	*A. suis*	*A. urinaehominis*
Conventional tests			–	+	–	–	–	+
	PYRA	+	+	+	+	ND	ND	–
	LAP	–	–	d	–	ND	ND	–
	BE	v	–	+	+	ND	ND	+
	NaCl 6.5 %	+	+	+	+	ND	+	+
	Hippurate	v	+	–	–	ND	–	+
	VP	–	+	–	–	ND	ND	–
Sugar fermentation								
	Maltose	v	–	–	+	+	–	–
	Mannitol	v	–	+	d	v	–	d
	Ribose	v	–	+	+		–	+
	Sucrose	v	–	+	–	+	–	–
	Trehalose	v	–	+	d	+	–	+
	Esculin	+	–	+	–		–	–
	D-Arabitol	–	–	–	d	ND	–	–
	Lactose	+	–	–	+	v	–	–
	Mannitol	v	–	–	+	ND	ND	–
	Maltose	+	–	+	–	+	–	+
	MBDG	v	–	d	–	ND		+
	Ribose	v	–	–	v	ND	+	+
	Sorbitol	v	–	–	v	+	–	–
	Sucrose	+	–	+	–	+	–	–
	Trehalose	+	–	+	–	+	–	–
Production during fermentation of:								
	β-GLUR	–	–	–	+	ND	ND	+
	PYR	v	–	+	–	ND	+	–

Note Symbols + >85 % positive; *v* variable between strains (16–84 % positive); – 0–15 % positive. *ND* no data available. *Abbreviations PYRA* pyrrolidonyl arylamidase; *LAP* leucine amine amino peptidase; *BE* Bile-esculin; *MBDG* methyl β-D-glucopyranoside; *β-GLUR* β-glucuronidase; *PYR* pyroglutamic acid arylamidase; *VP* Voges-Proskauer; for conventional tests see Facklam and Elliott (1995) and for API rapid ID32 Strep system tests see (Hammes and Hertel 2009)

(Shaw and Harding 1985). However, comparative 16S rRNA gene sequence analysis of these species and the genus *Carnobacterium* (Wallbanks et al. 1990) confirmed their similarity with *Carnobacterium* species and their distinction from all other LAB. Although *Carnobacterium* species were originally isolated with *Lactobacillus* species, phylogenetically, the genus is more closely related to the genera *Enterococcus* and *Vagococcus*. *Carnobacterium piscicola* [previously *Lb. piscicolu*, (Hiu 1984)] showed 100 % rRNA sequence homology with *Lb. mulfuromicus* (Miller et al. 1974), hence Collins et al. (1991) proposed that the correct name for both these organisms should be amended to *C. armltarornicus*. Currently, the genus *Carnobacterium* comprises 11 species with a variety of distinguishing physiological characteristics (Table 2.3).

Application in Food. Within the genera of LAB, *Carnobacterium* species are one of the most frequently isolated from natural environments and food and play a major role in biopreservation of food products. *Carnobacterium maltaromaticum* strains are widely found in foods including dairy products (Afzal et al. 2010) and have the potential to be applied as a protective culture in foods. Most research has focused on the production of bacteriocins by *C. maltaromaticum* and their roles in inhibition of *Listeria monocytogenes* and regulation of metabolic pathways of sensory importance (Leisner et al. 2007).

2.2.1.3 The Genus *Enterococcus*

Although species in the genus *Enterococcus* have been recognised since Thiercelin (1899) who described them as ‘entérocoque’ to emphasise their intestinal origin (Thiercelin 1899), the genus was not formally established until it was distinguished from the genus *Streptococcus* based on DNA–DNA and DNA–rDNA hybridisation studies (Collins et al. 1984). The first *Enterococcus*-type organism was described by Andrewes and Horder (1906) as *Streptococcus faecalis*, which was isolated from a patient with endocarditis (Andrewes and Horder 1906). Because there are no phenotypic characteristics to separate the genus from other genera of Gram-positive, catalase negative cocci, the taxonomy of this group of bacteria was vague until molecular tools became available. Application of molecular techniques for differentiation has resolved many of the uncertainties about these bacteria. However, the physical and chemical characteristics of growth at different temperatures, carbohydrate fermentation type and cell wall peptidoglycan type remain effective for distinguishing between *Enterococcus* species (Table 2.4).

Application in Food. Strains of *Enterococcus* species are mainly used in pig and poultry nutrition. However, there are pharmaceutical products that contain *Enterococcus* species as a probiotic. The genus *Enterococcus* contains 46 species (Table 2.4), but only *E. faecalis* and *E. faecium* are used as probiotics for animals and humans, of which *E. faecalis* is primarily used as a human probiotic.

Table 2.3 Physiological properties of species in the genus *Carnobacterium*[a] used for identification and differentiation

Characteristic	*C. alterfunditum*[b]	*C.divergens*[b]	*C. funditum*[b]	*C. gallinarum*[b]	*C. iners*[b]	*C. inhibens*[b]	*C. jeotgali*[b]	*C. maltaromaticum*[b]	*C. mobile*[b]	*C. pleistocenium*[c]	*C. vindans*[d]
Growth at different temperatures											
Growth at 0 °C	+	+		+	+	+	ND	v	+	+	2 °C(+)
Growth at 30 °C	–	+	–	ND	–	–	+	+	+	–	+
Growth at 40 °C	–(+)	+	–(+)	ND	–	–	+	v	–	–	–
Motility	+	–	+	–	ND	+	ND	–	+	+	–
Arginine hydrolysis	+	+	–	+	ND	+	–	–	+	ND	–
Voges-Proskauer test	ND	+	ND	+	ND	ND	ND	+	–	ND	–
Sugar fermentation											
Amygdalin	+	+	–	+	ND	+	–	v	–	ND	–
Arabinose	–	–	–	–	w	–	–	–	–	+	–
Galactose	w	–	w	+	ND	+	–	+	+	ND	+
Gluconate	–	+	-[d]	+	ND	–	–	+	–	ND	–
Glycerol	W	ND	w	ND	–	–	–	+	+/–	–	–
Inulin	–	–	–	–	ND	w	–	+	+	ND	–
Lactose	–	–	–	+	+	w	–	–	–	+	+
Mannitol	–	–	+	–	+	+	+	+	–	+	–
Melezitose	–	d	–	+	ND	–	–	v	–	ND	–
Melibiose	–	–	–	–	ND	–	–	+	–	ND	–
Methyl D-Glucoside	-[d]	–	–	+	ND	–	ND	+	–	ND	–
Ribose	+	+	+	+	+	+	–	+	+	+	+
D-Tagatose	ND	–	ND	+	ND	–	–	–	v	ND	+
Trehalose	–	+	+	+	+	+	–	+	+	+	+
D-Turanose	–	–	–	+	ND	–	ND	v	–	ND	–
Xylose	–	–	–	+	ND	–	–	–	–	ND	–
Esculin hydrolysis	+	ND	–	+	+	+	+	+	–	–	ND

Note [a] *Symbols* + positive; – negative; *v* variable (11–89 % positive); *w* weak; *ND* no data available
[b] The table is compiled from the studies of Second edition of Bergey's Manual of Systematic Bacteriology 2 rnd, Vol (3) Hammes and Hertel (2009)
[c] Data from Kim et al. (2009)
[d] Data from Pikuta et al. (2005)

Table 2.4 Physiological characteristics of species in the genus *Enterococcus* used for identification and differentiation

Characteristics	*E. avium*	*E. alcedinis*	*E. asini*	*E. aquimarinus*	*E. caccae*	*E. camelliae*	*E. canis*	*E. canintestini*	*E. casselifavus*	*E. cecorum*	*E. columbae*	*E. devriesei*
Motility	–	–	–	–	–		–	–	+	–	–	
Growth at different temperatures												
45 °C	+	–	±	+	+	+	+	ND	+	+	ND	–
50 °C	–	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND
Growth in												
6.5 % NaCl	+	–	–	(+)	+	+	+	+	+	–	–	+
0.04 % nitrite	–	ND	ND	ND	–	ND	ND	ND	+	ND	ND	ND
0.01 % tetrazole	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
Methylene blue (0.1 % milk) test:	d	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pigment production	–	ND	–	ND	–	ND	ND	–	+	d	ND	ND
Haemolysis	α	ND	ND	+	ND	Weak	ND	ND	ND	α	–	ND
H2S	+	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND
NH3 from arginine	ND	–	ND	–	–	–	+	ND	+	–	ND	–
Arginine dehydrolase	–	–	–	–	–	–	+	ND	+	–	ND	–
Hippurate hydrolysis	d	–	+	–	–	ND	+	ND	–	–	ND	–
Voges-Proskauer test	–	+	ND	–	+	ND	+	ND	+	+		+
Sugar fermentation												
D–xylose	–	+	+	+	ND	–	ND	–	+	–	+	–
L–rhamnose	+	–	+	–	ND	–	ND	–	(+)	+	+	–
Sucrose	+	+	–	+	+	+	+	+	+	+	–	+
Lactose	+	–	+	+		–	–	+	+	+	+	+
Melibiose	–	–	–	+	–	–	–	–	+	+	+	–
Raffinose	–	–	–	+	–	–	–	–	+	+	+	–
Melizitose	+	ND	–	–	ND	–	–	–	+	+	ND	–
Glycerol	+	–	–	–	+	–	+	–w	–	–	–	–

(continued)

Table 2.4 (continued)

Characteristics		*E. avium*	*E. alcedinis*	*E. asini*	*E. aquimarinus*	*E. caccae*	*E. camelliae*	*E. canis*	*E. canintestini*	*E. casselifavus*	*E. cecorum*	*E. columbae*	*E. devriesei*
	Adonite	+	ND	ND	ND	ND		ND	ND	–	–	ND	–
	Sorbitol	+	–	–	–	–	–	–	–	–	–	+	–
	Mannitol	+	+	–	–	–	+	–	–	+	+	+	–
Lancefield		Q (D)	D	D	–	D	ND	D	Non-D	D	Non-D	D	
Reference		Collins et al. (1984)	Frolkova et al. (2013)	De vaux et al. (1998)	Švec et al. (2005a)	Carvalho et al. (2006)	Sukontasing et al. (2007)	De graef et al. (2001)	Naser et al. (2005)	Collins et al. (1984)	Williams et al. (1989)	Devriese et al. (1990)	Švec et al. (2005b)

Characteristics		*E. dispar*	*E. durans*	*E. eurekensis*	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. gilvus*	*E. haemoperoxidus*	*E. hermanniensi*	*E. hirae*	*E. italicus*
Motility		–	–	–	(–)		–	–	–		–	–
Growth at different temperatures												
	45 °C	–	+	+	+	+	+	+	ND	–	+	v
	50 °C	–	–	ND	(–)	(–)	+	ND	ND	ND	+	ND
Growth in												
	6.5 % NaCl	+	+	+	+	+	+	+	ND	+	+	–
	0.04 % nitrite	ND	–	ND	+	–	(+)	ND	ND		–	ND
	0.01 % tetrazole	ND	–	ND	+	–	+	+	ND		ND	ND
Methylene blue (0.1 % milk) test:		ND	+	ND	ND	ND	v	ND	ND	ND	ND	ND
Pigment production		–	–	ND	ND	–	–	+	ND		ND	–
Haemolysis		ND	α, β	ND	(β)	(α)	α, β	ND	ND	α	–	α
H2S		–	–	ND	ND	ND	–	ND	ND		ND	ND
NH3 from arginine		–	ND	+	+	+	+	ND	ND	ND	ND	ND
Arginine dehydrolase		+	+	+	+	+	+	ND	–	ND	+	–
Hippurate hydrolysis		d	d	–	–	+	+	ND	–	+	+	+
Voges-Proskauer test		ND	ND	–	–	ND	ND	ND	ND	ND	+	ND

(continued)

Table 2.4 (continued)

Characteristics		E. dispar	E. durans	E. eurekensis	E. faecalis	E. faecium	E. gallinarum	E. gilvus	E. haemoperoxidus	E. hermanniensi	E. hirae	E. italicus
Sugar fermentation												
	D–xylose	–	–	–	–	–	+	–	–	–	–	–
	L–rhamnose	–	–	ND	d	–	–	+	–	-w	–	–
	Sucrose	+	–	ND	+	v	+	+	+		+	+
	Lactose	+	+	ND	+	+	+	+	+	–	+	+
	Melibiose	+	–	ND	–	v	+	+	–	ND	+	–
	Raffinose	+	–	ND	–	–	+	+	–	ND	+	–
	Melizitose	ND	–	ND	(+)	–	–	ND	ND	ND	(-)	–
	Glycerol	+	–	ND	+	+	+	+	+	ND	(-)	–
	Adonite	ND	–	ND	–	–	–	ND	ND	ND	–	ND
	Sorbitol	+	–	ND	(+)	–	–	+	–	–	–	–
	Mannitol	–	(-)	ND	+	(+)	+	+	v	+	–	v
Lancefield		Non-D	D	ND	D	D	D	D	ND	D	D	ND
Reference		Collins et al. (1991)	Collins et al. (1984)	Cotta et al. (2013)	Collins et al. (1984)	Collins et al. (1984)	Collins et al. (1984)	Tyrrell et al. (2002)	Švec et al. (2001)	Koort et al. (2004)	Farrow and Collins (1985)	Fortina et al. (2004)

Characteristics		E. lactis	E. lemanii	E. malodoratus	E. moraviensis	E. mundtii	E. pallens	E. phoeniculicola	E. plantarum	E. pseudoavlum	E. quebecensis	E. raffinosus
Motility		–	–	–	–	–	–	–	–	–	–	–
Growth at different temperatures												
	45 °C	+	+	–	–	+	+	–	+	+	–	+
	50 °C	ND	ND	–	–	–	ND	–	ND	ND	ND	ND
Growth in												
	6.5 % NaCl	+	+	+	+	+	+	–	+	–	–	+
	0.04 % Nitrite		ND	–	–	–	ND	ND	ND	ND	ND	ND
	0.01 % Tetrazole		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Methylene blue (0.1 % milk) test		+	ND		ND		ND	ND	ND	ND	ND	ND

(continued)

Table 2.4 (continued)

Characteristics		*E. lactis*	*E. lemanii*	*E. malodoratus*	*E. moraviensis*	*E. mundtii*	*E. pallens*	*E. phoeniculicola*	*E. plantarum*	*E. pseudoavlum*	*E. quebecensis*	*E. raffinosus*
Pigment production			–		–		+	ND	ND	ND	ND	ND
Haemolysis		α		ND	ND	–	ND	–	ND	α	ND	ND
H2S		ND		+	+	–	ND	–	–	ND		ND
NH3 from arginine		ND	ND		ND	ND	+	–	–			
Arginine dehydrolase		+	–		–	ND	–	ND	–			
Hippurate hydrolysis		v	d		d	ND	+	–	–		+	+
Voges-Proskauer test		+	–		–	ND	ND	ND	+		–	–
Sugar fermentation						ND						
	D-xylose	–		d	d	+	+	+	–	ND	ND	ND
	L-rhamnose	–	+w	+	+	(+)	ND	+	+	ND	ND	ND
	Sucrose	–	+w	+	+	+	+	+	–	–	+	+
	Lactose	+	+w	+	+	+	+	+	–	+	+	+
	Melibiose	+	+	+	+	+	+	–	–	–	+	+
	Raffinose	–	+	+	+	+	+	–	–	–	–	+
	Melizitose	–	ND	–	–	–	ND	–	ND	–	–	ND
	Glycerol	–		d	d	d	+	+	–	–	ND	+
	Adonite	ND		+	+	–	ND	ND	ND	ND	ND	ND
	Sorbitol	–		+	+	v	+	+	–	+	–	+
	Mannitol	+		+	+	+	+	–	–	+	+	+
Lancefield		ND		D	D	D	D	ND	ND	ND		(D)
Reference		Morandi et al. (2012)	Cotta et al. (2013)	Collins et al. (1984)	Švec et al. (2001)	Collins et al. (1986)	Tyrrell et al. (2002)	Law-Brown and Meyers (2003)	Švec et al. (2012)	Hammes and Hertel (2009)	Sistek et al. (2012)	Collins et al. (1989)

Characteristics			*E. ratti*	*E. rivorum*	*E. rotai*	*E. saccharolyticus*	*E. seriolicide*	*E. sulfureus*	*E. termitis*	*E. thailandicus*	*E. ureilyticus*	*E. viikkiensis*	*E. villorum*
Motility			–	–		–	–	–	–	–	–	–	–
Growth													
	at different temperatures												
		45 °C	+	–	–	+	+	+	+	+	+	–	+
		50 °C	ND	ND	ND	–	–	ND	ND	ND	ND	ND	ND

(continued)

Table 2.4 (continued)

Characteristics		*E. ratti*	*E. rivorum*	*E. rotai*	*E. saccharolyticus*	*E. seriolicide*	*E. sulfureus*	*E. termitis*	*E. thailandicus*	*E. ureilyticus*	*E. viikkiensis*	*E. villorum*
Growth in												
	6.5 % NaCl	+	–	–	+	+	+	+	+	–	+	+
	0.04 % Nitrite	ND	ND	ND	ND	+	ND	+	ND	ND	ND	ND
	0.01 % Tetrazole	ND	ND	ND	ND	+	ND	–	ND	ND	ND	ND
Methylene blue (0.1 % milk) test		–	ND	ND	+	ND	ND	ND	ND	ND	ND	
Pigment production		–	ND	ND	–	ND	+	–	ND		–	
Haemolysis		α	ND	ND	–	α	ND	–	+	α	α	α
H2S		ND	ND	ND	ND	–	ND	ND		ND	ND	ND
NH3 from arginine		+	+	+	–	ND	ND	ND	+	ND	ND	
Arginine dehydrolase		+	–	+	–	ND	–	–	ND	ND	–	
Hippurate hydrolysis		–	–	+	+	ND	–	–	ND	ND	–	
Voges-Proskauer test		ND	+	+	+	ND	ND	–	ND	ND	ND	
Sugar fermentation												
	D-xylose	+	v	–	–	–	+	+	ND	ND	–	D-
	L-rhamnose	ND	–	–	–	–	+	–	–	–	–	+
	Sucrose	–	+	+	+	–	+	–	+	–	ND	–
	Lactose	–	v	+	+	–		–	+	+	+	+
	Melibiose	–	–	+	+	–		–	–	–	–	+
	Raffinose	–	–	–	+	–	+	–	–	–	–	–
	Melizitose		–	+	+	–		–	ND	ND	–	ND
	Glycerol	–	+	+	–	–	–	+	+	+	+	+
	Adonite	ND	ND	ND	–	–	ND	ND	ND	ND	ND	ND

(continued)

Table 2.4 (continued)

Characteristics		*E. ratti*	*E. rivorum*	*E. rotai*	*E. saccharolyticus*	*E. seriolicide*	*E. sulfureus*	*E. termitis*	*E. thailandicus*	*E. ureilyticus*	*E. viikkiensis*	*E. villorum*
	Sorbitol	–	+	–	+	+	–	–	–	–	–	+
	Mannitol	+	+	+	+	+	+	–	+	–	+	–
Lancefield		D	Non-D	D	Non-D	Non-D	ND	ND	ND	D	D	ND
Reference		Teixeira et al. (2001)	Niemi et al. (2012)	Sedláček et al. (2013)	Chen et al. (2013)	Kusuda et al. (1991)	Martinez-Murcia and Collins (1991b)	Švec et al. (2006)	Tanasupawat et al. (2008)	Sedláček et al. (2013)	Rahkila et al. (2011)	De graef et al. (2001)

Note Symbols + positive; − negative; *v* variable (11–89 % positive); *w*, weak; *ND*, no data available

2.2.1.4 The Genus *Lactobacillus*

The genus *Lactobacillus* is most closely related to the genera *Paralactobacillus* and *Pediococcus*, being grouped within the same family (Release 5.0, Garrity et. al. 2004). The genus was first described by Beijerinck (1901). Then Orla-Jensen (1919) divided it into three subgenera, *Thermobacterium*, *Streptobacterium* and *Betabacterium* according to their optimal growth temperatures and hexose fermentation pathways. In the 2nd edition of Bergey's Manual of Systematic Bacteriology (Kandler and Weiss 1986), numerous *Lactobacillus* species were listed, and the nomenclature reorganised into three groups: group I (obligate homofermentative species), group II (facultative heterofermentative species) and group III (obligate heterofermentative species). This division suited the interests of food microbiologists; several species in groups I and II, and also some species in group III are used in fermented foods, although group III species are actually most commonly associated with food spoilage. Based on 16S rRNA the principal groupings are: (1) *Lb. delbrueckii* group including primarily the homofermentative species; (2) the *Lb. case*-*Pediococcus* group, comprised of obligate homofermentive species as well as facultative and obligate heterofermentive species; (3) the *Leuconostoc* group that includes some obligate heterofermentative species and has subsequently been subdivided into three genera: *Leuconostoc*, *Oenococcus* and *Weissella* (Collins et al. 1991). More recently the smaller groups have been split again according to their metabolic characteristics, phylogenetic groupings, guanine–cytosine content and the type of peptidoglycan present in the cell wall (Hammes and Hertel 2003). These detailed grouping of species was again updated by Felis and Dellaglio (2007) resulting in 106 validly described species separated into 15 groups some of which contained only single species (Felis and Dellaglio 2007). These species groups are *Lb. delbrueckii* group (delb), *Lb. salivarius group* (sal), *Lb. reuteri* group (reu), *Lb. buchneri* group (buch), *Lb. alimentarius*–*Lb. farciminis* group (al-far), *Lb. casei* group (casei), *Lb. sakei* group (sakei), *Lb. fructivorans* group (fru), *Lb. coryniformis* group (cor), *Lb. plantarum* group (plan), *Lb. perolens* group (per), *Lb. brevis* group (bre), *Pediococcus dextrinicus* group (Pdex) (Felis and Dellaglio 2007). In 2012, the taxonomy of the genus *Lactobacillus* was further updated and species within the genus were further clustered from a taxonomic point of view (Elisa et al. 2012). This updated phylogenetic analysis, also based on 16S rRNA gene sequencing, revealed 152 validly described species divided into 15 groups that contained three or more species, 4 groups that contained only two species and 10 single lines of descent (Elisa et al. 2012) (Table 2.5).

2.2.1.5 The Genus *Lactococcus*

The phylogenetic position of the genus *Lactococcus* within the *Firmicutes* was established by comparison of 16S rRNA gene sequences (Schleifer and Ludwig 1995a, b) and in the second edition of Bergey's Manual of Systematic Bacteriology

Table 2.5 Physiological characteristics of species in the genus *Lactobacillus* (validly published from 2012 to 2013) used for identification and differentiation

Characteristics	*Lb. backii*	*Lb. brantae*	*Lb. curieae*	*Lb. delbrueckii* subsp. jacobsenii	*Lb. delbrueckii* subsp. *sunkii*	*Lb. futsaii*	*Lb. heilongjiangensis*	*Lb. gigeriorum*	*Lb. kunkeei*	*Lb. hokkaidonensis*	*Lb. hominis*
Growth at											
15 °C	+	ND	+	+	–	+	ND	ND	+	–	–
45 °C	–	–	+	+	+	–	ND	+	–	–	+
PH 4.0	+		+	+	+	+	ND	+	–	+	–
PH 8.5	ND		w	ND	ND	+	ND	–	–	–	–
Salt tolerance 5 %	+	ND	+	ND	ND	ND	ND	–	ND	ND	+
Enzyme activity											
Alkaline phosphatase	–		ND	ND	ND		ND	+	ND	–	w
C4 esterase	+	ND	ND	ND	ND		ND	–	ND	+	–
C8 esterase	–	ND	ND	ND	ND		ND	–	ND	ND	–
Cystine aminopeptidase	–	ND	ND	ND	ND		ND	–	ND	ND	–
Naphthol–AS–BI–phosphohydrolase	–	ND	ND	ND	ND		ND	ND	ND	+	w
β-Galactosidase	–	–	ND	ND	ND		ND	–	ND	+	+
α-Galactosidase	–	ND	ND	ND	ND		ND	–	ND	+	+
β-Galactosidase	–	–	ND	ND	ND		ND	–	ND	+	+
N-Acetyl-β-glucosaminidase		ND	ND	ND	ND		ND	–	ND	+	–
Voges–Proskauer	ND	–	ND	ND	ND		ND		ND	–	+
Isomers of Lactic acid	D/L	ND	D/L	DL	DL	D/L	ND	L	D/L		DL
Sugar fermentation											
Aesculin	–	–	w	ND	ND	+	+	+	–	–	+
Amylose	ND	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-Arabitol	–	–	–	ND	ND	–	–	–	–	+	–
Arbutin	–	–	–	–	–	+	–	+	–	–	–
L-Arabinose	–	–	+	ND	ND	–	–	–	–	+	–
Cellobiose	–	+	–	+	–	+	+	+	–	–	+
D-Galactose	–	+	+	–	–	+	+	+	+	–	+
D-fructose		+	+	+	ND	+	+	ND	+	–	+

(continued)

Table 2.5 (continued)

Characteristics	*Lb. backii*	*Lb. brantae*	*Lb. curieae*	*Lb. delbrueckii* subsp. jacobsenii	*Lb. delbrueckii* subsp. *sunkii*	*Lb. futsaii*	*Lb. heilongjiangensis*	*Lb. gigeriorum*	*Lb. kunkeei*	*Lb. hokkaidonensis*	*Lb. hominis*
D-glucose	+	–	+	+	+	+	+	+	+	+	+
2-ketogluconate	–	ND	–		ND	ND	–	–	–	–	–
Lactose	–	–	w	–	–	+	+	–	–	–	+
Maltose	–	–(+)	+	+	+	+	+	+	–	+	+
D-Mannose	+	+	w	+	+	+	+	+	–	–	+
D-Mannitol	+	–	–	–	ND	–	–	–	+	–	–
Melezitose		–	+	ND	ND	–	–	–	–	–	–
D-Ribose	–	–	+	ND	ND	–	–	–	–	+	–
L-Rhamnose	–	–	–	ND	ND	–	–	–	–	–	–
Raffinose		–	+	–	–	–	–	–(–)	–	–	+
D-sorbitol	–	–	–		–		–	–	–	–	–
Salicin	–	–	–	–	+	+	+	+	–	–	+
Sucrose	–	–	+	+	+	+	+	+	+	–	+
D-xylose	–	–	–	ND	ND	–	–	–	–	+	–
Xylitol	–	–	–	ND	ND	–	–	–	–	–	–
G+C mol%	41.3	48.2 ± 2.6	44.1	50.2	50.9	36.3	38.9	41.9	36–37	39.4 ± 0.4	42.8
References	Tohno et al. (2013)	Dmitriy et al. (2012)	Lei et al. (2013)	David B et al. (2013)	Kudo et al. (2012)	Chao et al. (2012)	Gu et al. (2013)	Cousin et al. (2012)	Endo et al. (2012)	Tohno et al. (2013)	Cousin et al. (2013)

Characteristics	*Lb. iwatensis*	*Lb. kimchiensis*	*Lb. nasuensis*	*Lb. oryzae*	*Lb. pasteurii*	*Lb. porcinae*	*Lb. saniviri*	*Lb. shenzhenensis*	*Lb. senioris*	*Lb xiangfangensis*	*Lb yonginensis*
Growth at											
15 °C	+	+	ND	+	ND	+	ND	+	+	ND	+
45 °C	–	–	+	+	+	–	–	+	–	ND	–
PH 4.0	+	ND	w	+	+	+	+	+	–	ND	+
PH 8.5	–	+	ND	–	ND	ND	+	+	–	ND	–
Salt tolerance 5 %	+	+	–	+	ND	+	+	+	W	ND	+
Enzyme activity											
Alkaline phosphatase	–	ND	ND	+	+	ND	ND	ND	ND	ND	ND
C4 esterase	+	ND	ND	+	ND	ND	ND	ND	ND	ND	ND
C8 esterase	+	ND	ND	+	ND	ND	ND	ND	ND	ND	ND

(continued)

Table 2.5 (continued)

Characteristics	*Lb. iwatensis*	*Lb. kimchiensis*	*Lb. nasuensis*	*Lb. oryzae*	*Lb. pasteurii*	*Lb. porcinae*	*Lb. saniviri*	*Lb. shenzhenensis*	*Lb. senioris*	*Lb xiangfangensis*	*Lb yonginensis*
Cystine aminopeptidase	–	ND	ND	+	ND	ND	ND	ND	ND	ND	ND
Naphthol–AS–BI–phosphohydrolase	–	ND	ND	+	w	ND	ND	ND	ND	ND	ND
β-Galactosidase	–	ND	–	–	+	ND	ND	ND	ND	ND	ND
α-Galactosidase	–	ND	w	–	–	ND	ND	ND	ND	ND	ND
β-Galactosidase	–	ND	+	–	+	ND	ND	ND	ND	ND	ND
N-Acetyl-β-glucosaminidase	–	ND	ND	–	–	ND	ND	ND	ND	ND	ND
Voges–Proskauer	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND
Isomers of Lactic acid	DL	D/L	D/L	D/L	D	DL	DL	ND	D/L	ND	D/L
Sugar fermentation											
Aesculin	–		–	–	+	+	+	+	–	D	ND
Amylose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-Arabitol	–	ND	–	–	–	–	ND	–	–	–	+
Arbutin	–	w	–	–	+	–	+	+	+	–	–
L-Arabinose	–	w	w	–	–	–	–	+	+	–	+
Cellobiose	+	+	–	–	+	+	+	+	+	+	–
D-Galactose	–	+	+	+	–	–	+	+	–	+	+
D-fructose	+	+	+	–	+	+	+	+	+	ND	+
D-glucose	+	+	–		+	+	+	+	+	ND	+
2-ketogluconate	–	–	w	ND	ND	–	W	W	+	–	–
Lactose	–	+	–		+	–	–	W	–	–	ND

(continued)

Table 2.5 (continued)

Characteristics	*Lb. iwatensis*	*Lb. kimchiensis*	*Lb. nasuensis*	*Lb. oryzae*	*Lb. pasteurii*	*Lb. porcinae*	*Lb. saniviri*	*Lb. shenzhenensis*	*Lb. senioris*	*Lb xiangfangensis*	*Lb yonginensis*
Maltose	+	+	w		+	+	+	+	−	ND	+
D-Mannose	+	+	−	−	+	+	+	+	+	+	−
D-Mannitol	+	−	−	−	+	−	+	−	+	+	−
Melezitose	+	−	−	−	−	−	+	W	−	−	−
D-Ribose	−	−	+	−	+	−	+	W	+	ND	+
L-Rhamnose	−	−	−	−	−	−	−	+	+	−	ND
Raffinose		−	−	−	−	−	+	+	−	−	ND
D-sorbitol	−	−	−	−	−	−	−	−	−	+	ND
Salicin	−	+	−	−	+	+	+	+	+	+	+
Sucrose	−	+	−	−	+	+	+	+	+	+	−
D-xylose	−	−	w	−	−	−	ND	−	+	+	+
Xylitol	−	−	ND	−	−	−	+	−	ND	−	ND
G+C mol%	41.3	35.7	58.5–59.2	43.4	45.4	47.6	48.1	56.1-56.5	39.8	46.6	47.8
References	Tohno et al. (2013)	Kim et al. (2013)	Cai et al. (2012)	Tohno et al. 2013)	Cousin et al. (2013)	Nguyen et al. (2013)	Oki et al. (2012)	Zou et al. (2013)	Oki et al. (2012)	Gu et al. (2012)	Yi et al. (2013)

Note + 90 % or more strains positive; − 90 % or more strains negative; *d* 11–89 % of strains positive; *D* delayed reaction (positive after incubating at 30 °C for 4 days); *w* weak; *ND* no data available

(Hammes and Hertel 2009) *Lactococcus* was described as a member of the family *Streptococcaceae*, along with *Streptococcus* and *Lactovum*, in the order *Lactobacillales*. However, *Lactococcus* species are clearly separated from *Streptococcus* spp. which are pathogenic (Stackebrandt and Teuber 1988). To date, the genus *Lactococcus* comprises 12 species, amongst which, *Lactococcus lacits* including 3 subspecies. The species can be differentiated based on physiological characteristics (Table 2.6).

Application in Food. *Lactococcus lactis* subsp. *lactis* and *cremoris* are the species traditionally used in dairy applications. The main differences between the subspecies are their salt tolerance and ability to hydrolyse arginine. Both these are typical for subsp. *lactis* but absent in *cremoris*. The diacetyl-producing variants of *Lc. lactis* subsp. *lactis* are often referred to as biovar diacetylactis (Batt 2000).

2.2.1.6 The Genus *Leuconostoc*

Leuconostoc is the most economically important genus of LAB, and *Leuc. mesenteroide*s subsp. *mesenteroides* is the principle species isolated from plants (Mundt 1970). The *Leuconostoc* genus was considered close to the genus *Streptococcus* based on morphological classification criteria. Phenotypically, species from the *Leuconostoc*, *Lactobacillus* and *Pediococcus* genera share many characteristics and are often isolated from the same habitat (Garvie 1976; Sharpe 1972). Physiological properties can be used for identification and differentiation between species in *Leuconostoc* (Table 2.7). Phylogenetically, the genus *Leuconostoc* is closely related to the genera *Fructobacillus*, *Oenococcus* and *Weissella*, and together they are commonly known as the '*Leuconostoc* group' of LAB. Originally, the LAB included in the '*Leuconostoc* group' were all classified as *Leuconostoc* species. However, in the early 1990s, molecular phylogenetic analyses led to a subdivision of the group into three distinct lineages: the genus *Leuconostoc* sensu stricto, the *Leuconostoc paramesenteroides* group, and the *Leuconostoc oenos* group (Martinez-Murcia and Collins 1990; Martinez-Murcia et al. 1993). In the past 10 years some *Leuconostoc* species have been reclassified as *Oenococcus oeni* (Dicks et al. 1995) or moved to the genera *Weissella* (Collins et al. 1993) or *Fructobacillus* (Endo and Okada 2008) based on a combination of genetic and phenotypic characteristics. To date there are 16 validly described species (Table 2.6) with *Leuc. mesenteroides* being the type species (Euzeby 2009).

Application in Food. The role of *Leuconostoc* species in fermented food can be both positive and negative. The positive effects on dairy products were recognised early in the twentieth century when researchers found that during the fermentation process *Leuconostoc* species were responsible for a buttery aroma, which was a desirable characteristic for many dairy products (Thunell 1995; Dessart and Steenson 1995; Vedamuthu 1994). Currently, *Leuconostoc* species are used as starter cultures in the manufacture of fermented dairy, vegetable and cereal foods in conjunction with the acid-producing species *Lc. lactis*. The negative effects of

Table 2.6 Physiological properties of species in the genus *Lactococcus* used for identification and differentiation

Characteristics	*Lc. chungensis*	*Lc. garvieae*	*Lc. fujiensis*	*Lc. lactis*				*Lc. raffinolactis*	*Lc. piscium*	*Lc. plantarum*	*Lc. taiwanensis*
				cremoris	*hordniae*	*lactis*	*tructae*				
Growth at 4 °C	+	+	ND	–	+	+	+	–	+	–	ND
Growth at 10 °C	+	+	ND	+	+	+	+	+	ND	+	+
Growth at 45 °C	–	–	+	–	–	–	–	–	–	–	+
Growth at 4 % NaCl	–	+	–	–	–	+	ND	–	+	+	+
Growth at 6.5 % NaCl	ND		ND	ND	–	ND	ND	ND	ND	ND	+
Growth at pH 9.2	ND		ND	–	–	+	ND	ND	ND	ND	ND
Methylene blue (0.1 % milk)	ND	+	ND		–	ND	ND	ND	–	–	ND
Growth in Bile (40 %)	ND		ND	–	+	+	ND	ND	ND	ND	ND
NH_3 from arginine	+	+	ND	–	–	+	+	–	ND	ND	ND
CO_2 from citrate	ND		ND	–	+	–	ND	ND	ND	ND	ND
Diacetyl and acetoin	ND		ND	–		–	ND	ND	ND	ND	ND
Serological group[b]	ND		ND	N		N	ND	ND	ND	ND	ND
Sugar fermentation											

(continued)

Table 2.6 (continued)

Characteristics	*Lc. chungensis*	*Lc. garvieae*	*Lc. fujiensis*	*Lc. lactis*				*Lc. raffinolactis*	*Lc. piscium*	*Lc. plantarum*	*Lc. taiwanensis*
				cremoris	*hordniae*	*lactis*	*tructae*				
Galactose	–	+	+	+	–	+	+	+	–	–	+
Lactose	–	+	–	–	–	+	+	+	+	–	+
Mannitol	ND		+	–		–	+	ND	ND	+	+
Raffinose	–	–	ND	–	–	–	+	+	–	–	–
aesculin	+		ND	ND		–	ND	–	ND	+	ND
Maltose	+		ND	–	–	+	+	+	+	+	+
Melibiose	–		ND	–	–	–		+	+	+	–
Ribose	ND		+	–	–	+	+	ND	–	–	+
Starch	ND	–	+	–		–	ND	-w	ND	–	–w
DNA G+C (mol %)	ND	38.3–38.7	42.1–42.5	35.0–36.0	35.2	33.8–36.8	36	40–43	ND	36.9–38.9	39.6
References	Cho et al. (2008)	Schleifer et al. (1985)	Cai et al. (2011)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Schleifer et al. (1985)	Williams et al. (1990)	Schleifer et al. (1985)	Chen et al. (2013)

Note Symbols + positive; – negative; *w* weakly positive; *v* variable reaction; *ND* no data available

Table 2.7 Physiological properties of species in the genus *Leuconostoc* used for identification and differentiation

Characteristics	*Leuc. mesenteroides* subsp.				*Leu.carnosum*	*Leu.citreum*	*Leu.durionis*	*Leu. fallax*
	mesenteroides	*dexlranicm*	*cremoris*	*Leu.argentinum*				
Cell morphology	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid	Coccoid
Ammonia from arginine	–	–	–	–	–	–	–	–
Lactic acid configuration	D	D	D	D	D	D	D	D
Hydrolysis of esculin	+	+	–	–	v	+	–	ND
Dextran production	+	+	–	–	+	ND	ND	ND
Growth at Ph 4.8	–	–	–	ND	ND	ND	+	+
Growth in 10 % ethanol	–	–	–	ND	ND	ND	ND	ND
Growth at 37 °C	d	+	–	+	–	d	+	+
Requirement for TJE	–	–	–	–	–	–	–	–
G6PDH presente	+	+	+	ND	ND	ND	ND	ND
Sugar fermentation								
Amygdalin	ND	ND	ND	ND	ND	ND	–	ND
Arabinose	+	–	–	v	–	+	–	–
Arbutin	v	–	–	–	–	–	–	–
Cellulose	v	d	–	ND	ND	ND	ND	ND

(continued)

Table 2.7 (continued)

Characteristics	*Leuc. mesenteroides* subsp.				*Leu.carnosum*	*Leu.citreum*	*Leu.durionis*	*Leu. fallax*
	mesenteroides	*dexlranicm*	*cremoris*	*Leu.argentinum*				
Cellobiose	v	–	–	v	v	v	–	–
Lactose	v	+	d	+	–	–	–	–
Maltose	+	+	–	+-	–	+	–	–
Mannitol	v	–	–	v	–	d	+	(d)
Mannose	v	v	–	+		d	+	–
Raffinose	v	v	–	+	–	–	–	–
Ribose	v	+	–	–	d	–	+	+
Salicin	v	–	–	–	d	+	–	–
Sucrose	+	+	–	+	+	+	+	(+)
Trehalose	+	+	–	v	+	+	+	(+)
Xylose	v	d	–	v	–	–	–	–
Reference	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Dicks et al. (1993)	Shaw and Harding (1989)	Farrow et al. (1989)	Leisner et al. (2005)	Martinez-Murcia and Collins (1991a)

Characteristics	*Leu. ficulneum*	*Leu fructosum*	*Leu.gasicomitatum*	*Leuc.gelidum*	*Leu.inhae*	*Leu.kimchii*	*Leu.lactis*	*Leuc. pseudomesenteroides*
Cell morphology	Coccoid	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci	Coccoid or elongation of cocci
Ammonia from arginine	–	–	–	–	–	–	–	–
Lactic acid configuration	D	D	D	D	D	D	D	D
Hydrolysis of esculin	–	–	+	+	+	ND	–	d
Dextran production	+	–	+	+	v	+	–	ND

(continued)

Table 2.7 (continued)

Characteristics	*Leu. ficulneum*	*Leu fructosum*	*Leu.gasicomitatum*	*Leuc.gelidum*	*Leu.inhae*	*Leu.kimchii*	*Leu.lactis*	*Leuc. pseudomesenteroides*
Growth at Ph 4.8	–	ND	ND	ND	+	ND	–	ND
Growth in 10 % ethanol	ND	ND	ND	ND	ND	ND	–	ND
Growth at 37 °C	–	+	–	–	–	+	+	+
Requirement for TJE	–	–	–	–	–	–	–	-+
G6PDH presente	ND	ND	ND	ND	ND	ND	+	ND
Sugar fermentation								
Amygdalin	ND	ND	–	ND	v	+	ND	ND
Arabinose	–	–	+	ND	v	+	ND	ND
Arbutin	ND	ND	–	+	–	ND	–	ND
Cellulose	ND	ND	ND	ND	ND	ND	–	ND
Cellobiose	–	–	+	+	+	+	–	v
Lactose	–	–	–	–	–	+	+	v
Maltose	+	d	+	+	+	+	+	+
Mannitol	+	+	–	–	+	+	–	–
Mannose	+	–	–	+	+	+	d	+
Raffinose	–	–	+	+	–	+	d	v
Ribose	–	–	+	–	–	+	–	v
Salicin	ND	ND	–	+	d	+	d	v
Sucrose	–	+	+	+	+	+	+	v
Trehalose	+	–	+	+	+	+	–	+
Xylose	–	–	–	+	–	–	–	v
Reference	Antunes et al. (2002)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)	Hammes and Hertel (2009)

Note + positive; – negative; *w* weakly positive; *v* variable reaction; *ND* no data available

some *Leuconostoc* species is food spoilage. In 1878, a French natural scientist, Philippe van Tieghem studied slime-forming bacteria and described them as *Leuconostoc mesenteroides* (Euzeby 2009) and to date, *Leuconostoc* species are still implicated in the spoilage of packaged, refrigerated foods, particularly meat and meat products.

2.2.1.7 The Genus *Pediococcus*

Species in the genus *Pediococcus* were among the first bacteria to be studied by Louis Pasteur in relation to their role in the spoilage of beer. Their spherical shape and tetrad formation served as key characteristics in their early recognition. Initially, they were the only LAB that were divided in two planes to produce tetrads or pairs. However, more recent taxonomic changes have increased the number of tetrad forming genera to three. In Bergey's Manual of Systematic Bacteriology (Kandler and Weiss 1986) only 8 species were recognised in this genus but now there are 11 species that can be distinguished from each other based on physiological characteristics (Table 2.8). The species commonly referred to as *P. cerevisiae* was very variable and on subsequent study has been lost and the strains within it redistributed amongst the species *P. damnosus*, *P. acidilactici* and *P. pentosaceus*.

Application in Food. Most of the strains originally designated as *P. cerevisiae* that were used as meat starters have been reclassified as *P. acidiluctici*. Among the known *Pediococcus* species, *P. acidilactici*, *P. pentosaceus* and *P. halophilus* are most commonly associated with food fermentation.

2.2.1.8 The Genus *Streptococcus*

Species in the genus *Streptococcus* were amongst the earliest bacteria to be recognised by microbiologists because of their involvement in a large number of human and animal diseases. The generic name *Streptococcus* was first used by Rosenbach (1884) to describe the chain-forming, coccus-shaped bacteria associated with wound infections (Rosenbach 1884). The genus *Streptococcus* was originally described based on morphological, serological, physiological and biochemical characteristics and it comprised a wide range of organisms including the highly pathogenic bacteria *S. pneumoniae*, *S. pyogenes* and *S. agalactiae*; the intestinal group D *Streptococci S. faecalis* and *S. faecium*; and the economically important group N starter bacteria *S. cremoris* and *S. lactis* (these latter two species were subsequently placed in the genus of *Lactococcus*). There are more than 50 species in the *Streptococcus* genus and most are associated with human and animal disease, and therefore not of relevance to this chapter. Jones (1978) reviewed the composition and differentiation of the genus *Streptococcus* and proposed seven groups, including the strict anaerobes and pneumococci, based on pathogenicity, habitat and oxygen tolerance criteria (Jones 1978). However, based on molecular

Table 2.8 Physiological properties of species in the genus *Pediococcus* used for identification and differentiation

Characteristics		*P. acidilactici*	*P. damnosus*	*P. dextrinicus*	*P. halophilus*	*P. inopinatus*	*P. lolii* Doi et al. (2009)	*P. parvulus*	*P. pentosaceus*	*P. pentosaceus* subsp.*intermedius*	*P. stilesii*	*P. siamensis*	*P. urinaeeque*
Growth temperature	35 °C	+	–	+	+	+	+	+	+	+	+	ND	+
	40 °C	+	–	±(weak)	±(weak)	±(weak)	+	–	±(weak)	±(weak)	+	ND	±(weak)
	45 °C	+	–	±(weak)	–	–	–	–	±(weak)	±(weak)	+	+	±(weak)
	50 °C	+	–	–	–	–	–	–	–	–	ND	ND	–
Tolerance of NaCl		10 %	5 %	6 %	>18 %	8 %	ND	8 %	10 %	10 %	8 %	8 %	10 %
Growth at different pH													
	4.5	+	+	±	–	+	+	+	+	+	+		–
	5.0	+	+	+	–	+	+	+	+	+	+	+	–
	7.5	+	–	+	+	±	+	±	+	+	+	+	+
	8.0	+	–	–	+	–	+	–	+	+	+	+	+
	8.5	±	–	–	+	–	–	–	±	±	+	+	+
Catalase	–	–	–	–	–	–	–	–	±	±	–	–	±
CO_2 from glucose	–	–	+	–	–	–	–	–	–	–	–	–	–
Ammonia from arginine	+	–	–	–	–	–	ND	–	+	+	ND	–	–
Hippurate hydrolysis	–	–	–	–	–	–	ND	–	–	–	ND	ND	+
Formation of acetoin		±	±	–	±	–	ND	–	±	±	ND	ND	–
Configuration of Lactic acid		DL	DL	L(+)	L(+)	DL	DL	DL	DL	DL		DL	L(−)
Litmus milk reaction (0.1 % milk)	Acid	±	–	±	–	±	ND	–	+	+	ND	–	ND
	Reduction	±	–	±	–	±		–	+	+		–	ND
	Clotting	±	–	±	–	±		–	±	+		–	ND
Sugar fermentation													
	Arabinose	±	–	–	+	–	+	–	+	–	–	+	±
	Ribose	+	–	–	–	–	+	–	+	–	+	–	±
	Fructose	+	+	+	+	+	+	+	+	+	+	+	+
	Rhamnose	±	–	–	–	–	+	–	+	–	–	–	ND
	Glucose	+	+	+	+	+	ND	+	+	+	+	+	+
	Mannose	+	+	+	+	+	ND	+	+	+	+	+	+

(continued)

Table 2.8 (continued)

Characteristics	*P. acidilactici*	*P. damnosus*	*P. dextrinicus*	*P. halophilus*	*P. inopinatus*	*P. lolii* Doi et al. (2009)	*P. parvulus*	*P. pentosaceus*	*P. pentosaceus* subsp.*intermedius*	*P. stilesii*	*P. siamensis*	*P. urinaeeque*
Galactose	+	±	+	±	+	+	±	+	+	+	+	+
Maltose	−	±	+	+	+	−	±	−	+	+	−	+
Trehalose	±	±	±	+	+	−	+	+	±	+	+	±
Cellobiose	+	+	+	+	+	−	+	±	+	+	+	ND
Sucrose	±	±	±	−	−	−	−	−	±	−	−	+
Lactose	±	−	±	−	+	−	−	±	+	−	+	±
Melibiose	−	−	−	+	−	ND	−	±	±	−	ND	ND
Melezitose	−	±	−	±	−	−	−	−	−	ND	−	ND
Raffinose	±	−	−	−	−	−	−	−	±	−	−	+
Maltotriose	–	±	+	−	±	ND	±	±	±	ND	ND	ND
Dextrin	±	−	+	±	±	ND	±	±	−	ND	ND	±
Starch	−	−	+	−	−	ND	−	−	−	−	ND	−
Inulin	−	−	±	−	−	ND	−	−	±	−	ND	−
Glycerol	±	−	−	±	−	ND	−	−	±	+	−	−
Mannitol	±	−	−	−	−	ND	−	+	−	−	−	±
Sorbitol	−	−	−	+	−	ND	−	+	−	−	–	±
Salicin	−	±	±	+	±	+	−		−	+	+	ND
Amygdaloside	±	±	+	+	±	ND	+		±	ND	ND	+

Note + positive; − negative; *w* weakly positive; *v* variable reaction; *ND* no data available

studies, the genus has subsequently undergone major revisions. Similarities in 16S rRNA sequences from the Clostridium branch of gram-positive bacteria indicated that it was appropriate to separate the genus into three genetically distinct groups that were each genera in their own right: *Streptococcus* sensu stricto, *Enterococcus* and *Lactococcus* (Stackebrandt and Teuber 1988; Schleifer and Kilpper-BL lr 1984, 1987). The species remaining in the genus *Streptococcus* include all the pathogenic and the oral (Sherman's Viridans group) species. *Streptococcus thermopile* is an exception in this genus because it is the only one that is not a pathogen and is an important starter organism for yogurt and cheese manufacture.

Application in Food. *Streptococcus thermophilus* is widely used in combination with *Lb. delbrueckii* subsp. *bulgaricus, Lb. lactis* and/or *Lb. helveticus*, as a starter culture for yogurt and related fermented milk products as well as Swiss and Italian-type cheeses (Smit et al. 2005). It has an optimum incubation temperature above 40 °C. There is a special relationship between *S. thermophilus* and *Lb. delbrueckii* subsp. *bulguricus* in which *S. thermophilus* produces formic acid that promotes the growth of the other species which, in turn, provides flavour compounds (acetaldehyde) and the proteolytic activity to ensure that the *S. thermophilus* can grow in milk.

2.2.1.9 The Genus *Bifidobacterium*

According to the Taxonomic Outline of the Prokaryotes (Garrity et al. 2004), the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. The other genera in this family are: *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia* and *Scardovia*. Although, the genus *Bifidobacterium* is only poorly phylogenetically related to LAB and its species exploit a different metabolic pathway for the degradation of hexoses to the 'genuine' LAB, it has been listed amongst LAB in much of the traditional literature. In addition, species in the genus *Bifidobacterium* are as important as species in the genus *Lactobacillus* for food microbiology and human nutrition due to their role in food and feed production and preservation. Many species also exhibit probiotic properties. For these reasons, we include the genus *Bifidobacterium* in this chapter. The genus *Bifidobacterium* is comprised of 44 species and 9 subspecies (Table 2.9). Using a number of different methods and models for phylogenetic analyses, Felis and Dellaglio (2007) affirmed that, within the genus *Bifidobacterium*, there were distinct groups, each containing several associated species (Felis and Dellaglio 2007). These groups are: *B. adolescentis* group, *B. pullorum* group, *B. boum* group and *B. pseudolongum* group.

Table 2.9 Physical properties of species in the genus *Bifidobacterium* used for species identification and differentiation

Species	Sugar fermentation																			
	Sor-bitol	L-arabinos	Raf-finose	D-Ribose	Starch	Lactose	Inulin	Cello-biose	Mel-ezitose	Gluc-onat	Xylose	Man-nose	Fruc-tose	Galact-ose	Sucrose	Malt-ose	Treha-lose	Meli-biose	Manni-tol	Salicin
B. actinocoloniiforme[a]	–	+	–	+	–	ND	–	+	–	–	+	–	–	+	+	–	ND		–	+
B. adolescentis[b]	v	+	+	+	+	+	v	+	+	+	+	v	+	+	+	+	ND	–	d	+
B. angulatum[b]	d	+	+	+	–	+	+	–	–	v	+	–	+	+	+	+	–	–	–	–
B. animalis[b]	–	–	+	–	+	+	–	v	–	–	+	–	–	+	+	+	–	+	–	–
B. asteroids[b]	–	–	+	+	–	–	ND	+	–	v	+	–	+	v	+	v	–	+	–	+
B. biavatii	–	–	+	–	–	+	–	–	+	ND	+	–	w	+	+	+	ND	ND	+	+
B. bifidum[b]	–	–	–	–	–	+	–	–	–	–	–	–	+	+	d	–	–	+	–	–
B. bohemicum[c]	–	+	+	+	–ND	–	–	–	–	+	–	+	+	–	–	ND	ND	ND	–	+
B. bombi[b]	–	–	+	–	+	ND	+	–	–	ND	–	–	ND	w	ND	–	ND	ND	–	–
B. boum[b]	–	+	+	–	+	d	v	ND	v	–	–	–	+	+	+	+	v	+	–	v
B. breve[b]	v	–	+	+	+	+	v	v	v	–	–	+	+	+	+	+	v	–	v	+
B. callitrichos[d]	+	+	+	–	–	ND	–	+	+	ND	+	+	ND	+	+	+	ND	+	+	+
B. catenulatum[b]	+	+	+	+	+	+	v	+	–	v	+	–	+	+	+	+	v	+	+	–
B. choerinum[b]	–	+	–	–	+	–	–	ND	–	–	–	–	–	+	+	+	–	+	–	–
B. coryneforme[b]	–	–	+	+	–	–	v	+	+	+	+	–	+	ND	+	+	v	+	–	v
B. cuniculi[b]	–	+	+	+	+	v	–	–	v	–	+	+	+	+	+	+	–	+	–	–
B. dentium[b]	–	+	+	+	+	+	–	+	+	+	v	+	+	+	+	+	+	–	–	–
B. gallicum[b]	–	–	+	+	+	–	–	–	–	–	+	v	+	+	+	+	–	–	–	+
B. gallinarum[b]	–	+	+	+	–	–	+	–	–	–	+	+	+	+	+	+	+	+	–	+
B. indicum[b]	–	–	+	+	–	–	–	+	–	+	–	–	+	v	+	v	–	+	–	+
B. kashiwanohense[e]	+	+	+	+	–	+	–	+	–	ND	+	+	+	+	+	+	ND	ND	–	+
B.longum[b]	–	+	+	+	–	+	–	–	+	–	v	v	+	+	+	+	–	+	–	–
B.longum subsp. *infantis*[b]	–	–	+	+	–	+	v	–	–	–	v	v	+	+	+	+	–	+	–	–
B.longum subsp. *suis*[b]	–	+	+	+	–	+	+	v	v	+	+	v	+	+	+	+	+	+	–	+
B.magnum[b]	–	–	+	–	–	v	+	–	–	ND	+	–	+	+	+	+	–	+	–	–
B. merycicum[b]	–	+	+	+	+	+	–	v	–	–	+	–	+–	+	+	+	–	–	–	+–
B. minimum[b]	–	+	+	–	+	+	–	ND	–	–	–	v	v	–	+	+	–	+	–	–

(continued)

Table 2.9 (continued)

Species	Sugar fermentation																			
	Sor-bitol	L-arabinos	Raf-finose	D-Ribose	Starch	Lactose	Inulin	Cello-biose	Mel-ezitose	Gluc-onat	Xylose	Man-nose	Fruc-tose	Galact-ose	Sucrose	Malt-ose	Treha-lose	Meli-biose	Manni-tol	Salicin
B. mongoliense[b]	−	+	+	+	+	+	−	+	−	w	−	−	−	+	ND	−	ND	ND	−	+
B. pseudocatenulatum[b]	−	+	+	+	+	+	−	v	−	v	+	+	+	+	+	+	v	−	+	−
B. pseudolongum[b]	−	+	+	+	+	+	−	+	−	−	+	−	+	+	+	+	ND	ND	ND	±
B. pseudolongum subsp. *Globosom*[b]	−	v	+	+	+	+	−	−	−	−	+	−	+	+	+	+	−	+	−	−
B. psychraerophilum[f]	−	+	−	+	−	+	−	+	+	w	+	−	+	+	+	+	ND	ND	−	+
B. pullorum[b]	−	−	+	+	−	+	−		−	−	+	−	+	+	+	+	−	+	−	−
B. reuteri[d]	−	−	+	−	+	ND	−	w	w	ND	+	−	ND	−	ND	+	ND	w	−	+
B. ruminantium[b]	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+
B. saeculare[b]	−	+	+	+	−	-+	+−	−	+	−	+	−	+	+-	+	+	+	+	−	−
B. saguini[d]	−	+	+	+	−	+	−	+	−	w	+	−	+	+	+	+	ND	+	−	+
B. scardovii[b]	−	+	+	+	ND	ND	ND	ND	w	ND	ND	+	ND	−	ND	+	ND	−	−	ND
B. stellenboschense[d]	+	+	+	+	−	ND	−	−	+	ND	+	−	+	+	+	+	ND	ND	+	+
B. subtile[b]	+	+	−	−	+	−	−	−	−	+	−	−	+	+	+	+	−	ND	−	−
B. thermophilum[b]	−	−	+	+	+	+	−	v	v	−	−	v	+	+	+	+	v	+	−	+
B. thermacidophilum[b]	+	+	+	v	+	ND	v	ND	+	ND	v	−	ND	+	ND	ND	ND	+	−	+
B. tsurumiense[b]	−	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	ND	ND	+	+

Note + positive; − negative; *w* weakly positive; *v* variable reaction; *ND* no data available; [a] data from the references Killer et al. (2011); [b] data from the references Goodfellow (2012); [c] data from the references (Killer et al. 2011); [d] data from the references (Endo et al. 2012); [e] data from the references (Morita et al. 2011); [f] data from the references (Simpson et al. 2004)

2.2.2 Species of Lactic Acid Bacteria

Although more than 50 genera of LAB have been validly published till now, a considerable number of species have been described and increased sharply. The most important genera are described above, among which (alphabetically) *Enterococcus, Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* are regarded as the most important food microorganisms. Thus, the most important species in these genera are described in more detail in this section.

Species of *Enterococcus*. Bacteria from the genus *Enterococcus* were first observed by Thiercelin (1899) and Andrews and Horder (1906) used the name *S. faecalis* for the *Enterococcus*-type organism that they isolated from a patient with endocarditis. In 1937, Sherman first described those organisms from physiological and biochemical and serological characteristics (Devriese et al. 1993; Seegers 2002). To date, there were 45 validly described species in the genus *Enterococcus* from a range of different sources (Table 2.10).

Species of *Lactobacillus*. *Lactobacillus* is the largest group of LAB and includes some of the most important species involved in food microbiology and human nutrition; several *Lactobacillus* species are essential in fermented food production and are used as starter cultures or food preservatives. Moreover, certain species of human origin are being exploited as probiotics or vaccine carriers (Seegers 2002). This genus includes a large number of GRAS species (Generally Recognised As Safe). The number of species described has increased sharply in recent years (Fig 2.2). There were 74 new species of *Lactobacillus* described between the year of 2001 and 2010. This is the same number of new *Lactobacillus* species described in the whole of the twentieth century. This amazing increase in the number of *Lactobacillus* species identified has a strong relationship with advances in the development of innovative molecular techniques and their application to microbial taxonomy and identification. This was particularly evident in the 1980s when a large number of new species were identified using 16S rRNA gene sequence analysis and then again in the last 10 years due to the advent of polyphasic taxonomic methods.

Species of *Lactococcus* On the basis of exhaustive reinvestigations, N streptococci (Lancefield 1933) were separated from the oral streptococci, enterococci and hemolytic streptococci, and placed in a new genus, *Lactococcus*, by Schleifer et al. (1985, Lancefield (1933). In recent years, molecules have allowed detection and differentiation of a number of new species. However, the genus remains relatively compact and comprises only 12 species (Table 2.5). Of these, *Lactococcus lactis* is the most important in industrial fermentation and especially for the manufacture of dairy products. There are four subspecies of *Lc. lactis*: *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *hordniae*, *Lc. lactis* subsp. *tructae*. Subsp. *lactis* is the most important commercially used LAB.

Species of *Pediococcus* This genus was one of the first LAB to be studied intensively by food microbiologists because of their association with beer spoilage. Species in *Pediococcus* can be confused with micrococci and aerococci because of

Table 2.10 Detailed information of species of *Enterococcus*

Species	Source	Principal habitats	References
E. alcedinis	New	Common kingfisher	Frolkova (2013)
E. aquimarinus	New	Sea water	Švec et al. (2005a)
E. asini	New	Caecum of donkeys	De vaux (1998)
E. avium	Transfer	Poultry and mammalian intestines	Collins et al. (1984)
E. caccae	New	Human stools	Carvalho et al. (2006)
E. canintestini	New	Fermented tea leaves in	Naser et al. (2005)
E. canis	New	Faecal samples of healthy dogs	De graef et al. (2001)
E. casseliflavus	Transfer	Grass, silage, plants, soil	Collins et al. (1984)
E. cecorum	Transfer	Clinical origin, animals	Williams et al. (1989)
E. columbae	New	Pigeon intestines	Devriese et al. (1990)
E. devriesei	New	Animal sources	Švec et al. (2005b)
E. dispar	New	Human sources	Collins et al. (1991)
E. durans	Transfer	Clinical origin, animals	Collins et al. (1984)
E. eurekensis	New	Swine-manure storage pit	Cotta et al. (2013)
E. faecalis	Transfer	Human and other animal intestines	Schleifer and Kilpper-BHlz (1984)
E. faecium	Transfer	Human and other animal intestines, including poultry	Schleifer and Kilpper-BHlz (1984)
E. gallinarum	Transfer	Poultry intestines	Collins et al. (1984)
E. gilvus	New	Human clinical specimens	Tyrrell et al. (2002)
E. haemoperoxidus	New	Water	Švec et al. (2001)
E. hermanniensis	New	Modified-atmosphere-packaged broiler meat and canine tonsils	Koort et al. (2004)
E. hirae	New	Young chickens	Farrow and Collins (1985)
E. italicus	New	Italian cheeses	Fortina et al. (2004)
E. lactis	New	Italian raw milk cheeses	Morandi et al. (2012)
E. lemanii	New	Swine-manure storage pit	Cotta et al. (2013)
E. malodoratus	Transfer	Originally from Gouda cheese	Collins et al. (1984)
E. moraviensis	New	Water	Švec et al. (2001)

(continued)

Table 2.10 (continued)

Species	Source	Principal habitats	References
E. mundtii	New	Grass, silage, plants, soil	Collins et al. (1986)
E. pallens	New	Human clinical specimens	Tyrrell et al. (2002)
E. phoeniculicola	New	Uropygial gland of the Red-billed Woodhoopoe	Law-Brown and Meyers (2003)
E. plantarum	New	Plants	Švec et al. (2012)
E. pseudoavium	New		Collins et al. (1989)
E. quebecensis	New	Water	Sistek et al. (2012)
E. raffinosus	New	Clinical isolates, endocarditis	Collins et al. (1989)
E. ratti	New	Associated with enteric disorders in animals	Teixeira et al. (2001)
E. rivorum	New	Water of pristine brooks	Niemi et al. (2012)
E. rotai	New	Environment	Sedláček et al. (2013)
E. saccharolyticus	Transfer	Bedding and skin of cattle	Chen et al. (2013)
E. silesiacus	New	The gut of a termite	Švec et al. (2006)
E. solitarius	New		Collins et al. (1989)
E. sulfurous		Yellow-pigmented E. species	Martinez-Murcia and Collins (1991b)
E. termites	New		Švec et al. (2006)
E. thailandicus	New	Fermented sausage in Thailand	Tanasupawat et al. (2008)
E. ureasiticus	New	Water	Sistek et al. (2012)
E. ureilyticus	New	Environment	Sedláček et al. (2013)
E. viikkiensis	New	Broiler products	Rahkila et al. (2011)

Note new new species; *transfer* transfer from group D streptococci from Devriese and Pot (1995)

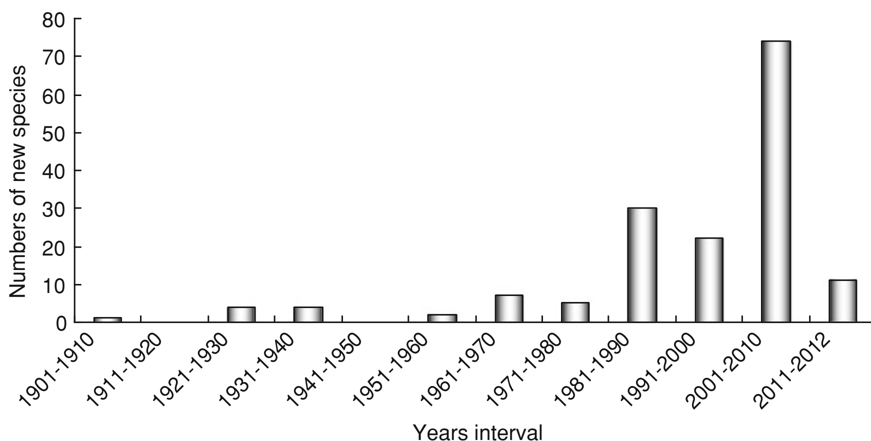


Fig. 2.2 Number of new species of *Lactobacillus* found between 1901 and 2012

morphological similarities, pseudocatalase production and salt tolerance (Garvie 1986). In Bergey's Manual of Systematic Bacteriology (Garvie 1986) eight species were recognised but the number of species has now increased to nine (Hammes and Hertel 2009). To date, there were 15 validly described species in the genus (http://www.bacterio.net/p/pediococcus.html).

Species of *Leuconostoc* *Leuconostoc* is the predominant genus among the LAB on plants, with *Leuc. mesenteroides* subsp. *mesenteroides* as the principal species (Mundt 1970). *Leuconostocs* are traditionally found in association with plant matter, fermented dairy products and wines. The first species was isolated in 1878 by Cienkowski. There were four species listed in the Bergey's Manual of Systematic Bacteriology (Garvie 1986) but, at the time of writing, there are now 12 species in the genus (Table 2.7).

Species of *Streptococcus* The genus *Streptococcus* is large, containing numerous clinically significant species that are responsible for a wide variety of infections in man and in animals. To date there are 82 approved listed species (http://www.bacterio.net/s/streptococcus.html).

2.3 Genetic Diversity of Lactic Acid Bacteria

2.3.1 Biodiversity of Lactic Acid Bacteria as Identified by Culture-Dependent Approaches

LAB have often been isolated and identified from traditionally fermented dairy products in the last 20 years. These fermented dairy products include busa (Turkestan), cieddu (Italy), dadhi (India), kefir (Balkans), kumiss (Steppes), kurut

(Himalayan regions), laban zabadi (Egypt), mazun (Armenia), taette (N. Europe), skyr (Iceland), masl (Iran), crowdies (Scotland), kuban and yogurt (Miyazaki and Matsuzaki 2008). Studies of the different types of raw milk used (including cow, goat, ewe, mare, camel, buffalo and yak) show a great diversity of LAB at the genus and species level. For example, 12 different species of LAB were isolated and identified during the manufacture and storage of Anevato cheese, a traditional spreadable Greek cheese made from raw goat's or ewe's milk: three species were from the genus *Lactococcus* (*Lc. lactis*, *Lc. garviae*, *Lc. raffinolactis*), three species were from the genus *Leuconostoc* (*Leuc. mesenteroides*, including the subspecies *mesenteroides* and *dextranicum*, *Ln. paramesenteroides* and *Leuc. lactis*) and six species were from the genus *Lactobacillus* (*Lb. plantarum*, *Lb. coryneformis*, *Lb. paracasei*, *Lb. brevis*, *Lb. bifermentans* and *Lb. viridens*).

2.3.1.1 LAB in Koumiss

Koumiss (also known as koumiss, kumiss, kumys, kumyz, kimiz or coomys) is a very popular drink consumed in countries from the Cancasus region of Kazakhstan, Azerbaijan, Turkey (kumye) and in China and Mongolia (Tooner 1994). In the seventh century, koumiss became an everyday drink of the Mongolian tribes where, today, the product is known as airag, arrag, irag, chige or chigo (in the Mongolian language) (Zhang and Zhang 2012). Traditional koumiss is produced from mare's milk and in Mongolia it is also produced from camel's milk. In Europe and North America, a koumiss-like product is made from full or skimmed cow's milk (Mann 1989; Di Cagno et al. 2004).

The microflora isolated from koumiss has not been well defined, but consist mainly of LAB and yeast. Indeed, *Saccharomyces unisporus* was identified as the principal microorganism from traditional koumiss in Kazakhstan (Montanari and Grazia 1997; Park et al. 2006). Sometimes, acetic acid bacteria have also been found (Zhang and Zhang 2012; Park et al. 2006). In recent years, numerous studies have been published clarifying the diversity of LAB isolated and grown in pure culture from traditional koumiss in China and Mongolia (Table 2.11).

2.3.1.2 LAB in Traditional Fermented Camel's Milk and Related Products

Camels (*Camelidae*) mainly inhabit desert and semi-desert regions, with major populations distributed within Sudan and other African countries, Arab countries, India, Mongolia and the northwest region of China (Jiri et al. 2009). Raw camel milk products, fresh unpasteurised milk or spontaneously fermented milk (such as suusac) are also an important component of the daily diet of pastoralists in East African countries such as Kenya and Somalia (Farah et al. 2007). In Sudan, fresh unpasteurised camel's milk and fermented camel's milk (gariss) are widely consumed by pastoralist communities living in the arid and semi-arid regions of the

Table 2.11 Composition and distribution of lactic acid bacteria in koumiss of different regions of Mongolia

Countdry/ Regions	Number of samples	Number of isolates	Species of LAB	Total	Predominant species	References
Mongolia	3	–	*Lb.helveticus*	–	***Lb. helveticus***	Uchida et al. (2007)
			Lb.kefiri			
			Lb.paracasei			
			Lb.plantarum			
			Lb.farciminis			
			Lb.curvatus			
	22	367	*Lb.helveticus*	93	***Lb. helveticus***	Watanabe et al. (2008)
			Lb. kefiranofaciens	35	***Lb. kefiranofaciens***	
			Lb.casei	13		
			Lb. diolivorans	1		
			Lb.farciminis	1		
			Lb.higardii	1		
			Lb.kefiri	4		
			Lb.parafarranginis	1		
			Lb.plantarum	8		
			Lc.lactis subsp.*lactis*	7		
			Leuc.mesenteroides	13		
			Leuc.pseudomesenteroides	2		
			Lb.fermentum	1		
			Lactococcus	2		
			S. thermophilus	1		
			E. faecium	1		
	5	30	*Lb. acidophilus group*	20	***Lb. acidophilus group***	Menghebilige et al. (2004)
			Lb. casei	1		

(continued)

Table 2.11 (continued)

Countdry/ Regions	Number of samples	Number of isolates	Species of LAB	Total	Predominant species	References
			Lb. plantarum	9		
			Lb. paracase subsp.*paracase*	–		Burentegusi et al. (2002)
			Lb. coryniformis subsp.*coryniformis*	–		
			Lb. curvatus			
			Lb. kefiranofaciens			

country (Ashmaig et al. 2009). Traditionally, fermented camel's milk and are also popular in China (*shubat* in the Hazakh language) (and Mongolia (*hogormag* in the Mongolian language) (Shuangquan and Miyamoto 2004). Early reports indicated that the bacterial component of the microflora of traditional fermented camel milk products was dominated by LAB species belonging to several different genera. In recent years, in order to improve the spontaneous traditional fermentation and develop suitable starter cultures for safer production of traditional food, many species of LAB have been isolated and identified from camel's milk by different research institutes and in different countries and regions (Table 2.12).

2.3.1.3 LAB in Traditional Fermented Cow's Milk and Related Products

Historically, fermented dairy products were developed by nomadic tribes people to preserve the valuable nutrients from fresh raw milk. With the advancement of human civilization and changes in life style, the role of these foods as the sole source of nutrition for nomadic people has ceased. However, the process of making these traditional fermented dairy products continues to be developed and handed down through the generations in the minority ethnic populations of China. Nowadays, many different fermented dairy products are consumed by minority ethnic people living in Mongolia, Xinjiang, Tibet and other provinces of China. However, as cows are currently the main domestic livestock variety in China, cow's milk is the major raw material for making a variety of these dairy products, including *dairy fan*, *yogurt*, *Eedsen Su Aarchi* and *wurum*. The Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education P.R.China have long been engaged in isolating and identifying LAB from products traditionally fermented from cow's milk in China. From 2001 to the present, a wide variety have been isolated and identified in a selection of different ethnic minority areas of China, Mongolia and Tibet (http://www.bio149.cn/html/labcc.html) (Table 2.13).

2.3.1.4 LAB in Kurut and Other Fermented Yak's Milk Products

Yaks are one of the most ancient bovine species in China and mainly distributed in the Qing–Tibetan plateau at an average altitude of 4,000 m. China has approximately 13 million yaks which is the largest number for any country in the world (Zhang et al. 2008). China has both the largest number of yak herds and the highest associated milk production in the world (Zhu and Zhang 2005). Many varieties of dairy products including kurut, qula, butter and cheese are prepared from yak's milk and consumed by Tibetan people in the Qing–Tibetan plateau regions of China. Kurut is one of the most important fermented products and is made using traditional production methods as reported by Chen et al. (2009). The high altitude and special climate of the Tibetan plateau represents a distinct ecological with a wide biodiversity. Such diversity of species, including a diversity of LAB, results

Table 2.12 Diversity of LAB in naturally fermented camel's milk products

Species of LAB	Number of isolates	Predominant species	Type of samples and country of origin
E. avium	1[d]	–	Fermented milk, China
E. durans	2[d]	–	Fermented milk, China
E. faecalis	–[b]; 2[d]	–	Shubat, India; fermented milk, China
E. faecium	–[b]; –[e]	***E. faecium***	Shubat, India; fermented milk, China
Lb. casei. subsp. *pseudoplantarum*	1[d]	–	Fermented milk, China
Lb. acidophilus	1[d]; –[e]	–	Fermented milk, China
Lb. brevis	1[a]; –[b]	–	Gariss, Sudan; Shubat India
Lb. casei subsp. *casei*	5[d]	–	Fermented milk, China
Lb. casei	–[e]	–	Fermented milk, China
Lb. curvatus	1[d]	–	Fermented milk, China
Lb. divergens	1[a]	–	Gariss, Sudan
Lb. farcimini	1[d]	–	Fermented milk, China
Lb. fermentum	1[a]; 7[c]; 1[d]	–	Gariss, Sudan
Lb. gasseri	1[a]	–	Gariss, Sudan
Lb. helveticus	-[b]; –[e]	–	Shubat, India; fermented milk, China
Lb. paracasei* subsp. *paracasei	64[c]	***Lb. paracasei* subsp. *paracasei***	Gariss, Sudan
Lb. paracasei	1[a]		Gariss, Sudan
Lb. plantarum	7[a]; 3[c]; 4[d]	***Lb. plantarum***[a]	Gariss, Sudan; fermented milk, China
Lb. rhamnosus	1[a]		Gariss, Sudan

(continued)

Table 2.12 (continued)

Species of LAB	Number of isolates	Predominant species	Type of samples and country of origin
Lb. sakei	–[b]	***Lb. sakei***	Shubat India
Lc. alimentarium	1[a]	–	Gariss, Sudan
lc. lactis	–[b]; 2[d]	–	Shubat, India; fermented milk, China
Lc. raffinolactis	6[a]	–	Gariss, Sudan
Lc. subsp.*cremoris*	–[e]	–	Fermented milk, China
Leuc. lactis	–[b]; –[e]	–	Shubat, India; fermented milk, China
Leuc. mesenteroides subsp. *dextranium*	2[d]	–	Fermented milk, China
Leuc. mesenteroides subsp. *mesenteroides*	1[d]	–	Fermented milk, China
W. hellenica	–[b]	–	Shubat, India

Note [a] Represent data from Ashmaig et al. (2009); [b] represent data from Rahman et al. (2009); [c] represent data from Sulieman et al. (2006); [d] represent data from Menghebilige et al. (2004); [e] represent data from Shuangquan et al. (2004)

Table 2.13 The species and number of isolations of LAB from traditionally fermented cow's milk products in different regions

Species of LAB	Inner Mongolia	Tibet	Yunnan Province, China	Tibetan regions of Gansu and Sichuan Provinces, China	Mongolia
E. durans	10	7	1	9	3
E. faecalis	6	2	–	–	1
E. faecium	5			1	
E. italicus	1				
Lb. acetotolerans	1				
Lb. brevis	4			4	3
Lb. casei	106			31	16
Lb. crispatus	1				
Lb. crustorum		1			
Lb. curvatus	1				
Lb. delbrueckii subsp. *bulgaricus*	4			11	129
Lb. diolivorans	68			1	15
Lb. fermentum	5	1	10	10	42
Lb. helveticus	153	2	56	88	169
Lb. hilgardii	4		1	1	
Lb. kefiranofaciens subsp. *kefiranofaciens*	3				
Lb. kefiranofaciens subsp. *kefirgranum*	9		5		
Lb. kefirgranum	10		8		
Lb. kefiri	27		1		7
Lb. parabuchneri	15				
Lb. plantarum	68	8	1	4	7
Lb. pontis	5				
Lb. reuteri	11				
Lb. rhamnosus	4				
Lc. garvieae		1	2		
Lc. lactis subsp. *cremoris*	6	3		6	
Lc. lactis subsp. *lactis*	132	9	4		6
Lc. raffinolactis	3			13	
Leuc. lactis	2	1		1	22
Leuc. mesenteroides	6				38
Leuc. mesenteroides subsp. *mesenteroides*	97	3		49	16
Leuc. pseudomesenteroides	5				
S. thermophilus	18			38	182

in a very specific microflora associated with yak's milk and its products (Li et al. 2002; Luo et al. 2005). These differences in biodiversity of bacteria are very important in forming the typical features of traditional fermented dairy products in different regions. Several studies have reported the species composition and biodiversity of LAB in yak's milk products from different regions of China including Tibet (Zhang et al. 2008; Airidengcaicike et al. 2010; Duan et al. 2008) and the provinces of Qihai (Sun et al. 2010), Gansun (Bao et al. 2012a) and Sichuan (Bao et al. 2012b). It was described in Table 2.14. A predominance of *L. helveticus* was recorded in qula and whey products from Gansu and Sichuan provinces, respectively. It also predominated in kurut from Gansu province. However, the predominated species in kurut from Sichuan, Qinghai and Tibet were *Lb. helveticus*, *S. thermophilus* and *Lb. fermentum*, respectively. Other species from the genera *Lactobacillus*, *Enterococcus*, *Lactococcus,* and *Leuconostoc* were also isolated at a low frequency. The number of identified species ranged from one to over 20 depending on the product. The overall distribution pattern showed that the predominant species and biodiversity of LAB in yak's milk products from the four geographically distant regions varied substantially.

2.3.2 Biodiversity of Lactic Acid Bacteria as Determined by Culture-Independent Approaches

Before 1990, isolation and growth of microorganisms in pure culture was the only method for their identification and characterisation. For this reason, early species composition studies and biodiversity analyses were mainly achieved based on culture-dependent methods. However, it is well known that less than 1 % of microorganisms can be cultivated in the laboratory (Ward et al. 1990; Engelen et al. 1998). Furthermore, the in vitro growth step can lead to underestimates in biodiversity analysis because slow-growing species or species present in low numbers may be out-competed by faster growing or more prevalent species (Hugenholtz et al. 1998). The emergence of cultivation-independent approaches has overcome these issues and provides unique opportunities to reveal the diversity of previously unculturable prokaryotic species by analysis of the ribosomal RNA (rRNA) genes of mixed microbial populations from environmental samples (Hugenholtz et al. 1998). This is far more representative of the real spectrum of microorganisms and their genes that are active in the habitat of choice. To comprehensively assess the microbial composition of dairy products and other fermented food, studies on the microbial composition of LAB have been clarified using culture-independent approaches.

Table 2.14 Species and numbers of isolates of LAB from fermented yak's milk and related products in regions where Tibetan tribes people live regions

Species of LAB	Sichuan province[a]					Gansu province[b]					Tibet[c]	Qinghai[c] province
	Kurut	Raw milk	Qula	Whey	Butter	Kurut	Raw milk	Qula	Whey	Butter	kurut	Kurut
E. hormaechei subsp. *steigerwaltii*			1									
E. durans	1							1		2	8	7
E. faecalis												5
E. faecium	1		1								4	1
Lb. brevis						1						
Lb. buchneri		1										
Lb. casei	7	1	7	1		17					24	
Lb. coryniformis subsp. *torquens*					**2***							
Lb. delbrueckii subsp. *bulgaricus*	5		4	2		11						23
Lb. diolivorans									1			
Lb. fermentum	1		5	2	1	6	1	1	1		**51***	3
Lb. helveticus	7	4	**22***	**8***		**50***	4	**23***	**10***		4	13
Lb. hilgardii					1				1			
Lb. kefiri	4								2			
Lb. plantarum		5						2				12
Lb. rapi	1											

(continued)

Table 2.14 (continued)

Species of LAB	Sichuan province[a]					Gansu province[b]					Tibet[c]	Qinghai[c] province
	Kurut	Raw milk	Qula	Whey	Butter	Kurut	Raw milk	Qula	Whey	Butter	kurut	Kurut
Lb. uvarum	4			1								
Lc. lactis subsp. *cremoris*	4	1						2			4	5
Lc. lactis subsp. *lactis*	5	1	1	1				8			3	15
Lc. raffinolactis	5	1	1	1				8				
Leuc. citreum	6	2	6	1			4	1	2			5
Leuc. lactis	5	13	3	2							1	8
Leuc. mesenteroides subsp. *mesenteroides*	4	**29***	19	8	1	21	2	1	5	1	3	4
S. thermophilus	**20***	1	7					4	7	**4***		**51***
W. cibaria			1									

* Represent the predominant species in a given product by region. **Bold** means the species is the predominated species in this samples or region
Note [a] Represented the data from Bao et al. (2012a); [b] Represented the data from represented the data from Bao et al., 2012a) [c] represented the data from Sun et al. (2010)

2.3.2.1 Microbial Diversity in Traditionally Fermented Dairy Products as Determined by Metagenomic Sequence Analysis

Culture-independent approaches have been used increasingly to determine the composition of complex microbial communities. These procedures have enabled the simultaneous characterisation of whole ecosystems and the identification of many species from these sources (Quigley et al. 2011). There is a vast amount of information held within the genomes of microorganisms that cannot be cultured in conventional ways and metagenomics techniques are one of the key technologies now used to access and investigate this potential. These techniques have detected all of the commonly cultured species, as well as some previously undetected microorganisms. In recent years, pyrosequencing has been used increasingly to study the diversity and dynamics of the bacterial populations of an Irish kefir grain and its corresponding fermented products (Dobson et al. 2011) and other fermented food (Humblot and Guyot 2009; Roh et al. 2010; Jung et al. 2011). Biodiversity of LAB in traditionally fermented dairy products (including fermented cow's milk, koumiss and kurut) in China, Mongolia and Russia are also currently being investigated using pyrosequencing technology in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, China (Inner Mongolia Agricultural University) to complement our previous extensive culture-dependent studies (Bao et al. 2012a, b). Although these data are not published a summary will be provided in the following sections.

Microbial Diversity in Traditionally Fermented Cow's Milk

Samples of fermented cow's milk were collected from the Xinjiang Uygur Autonomous Region, China (n = 10 from Zhaosu County and 12 from Tekesi County) and subjected to microbial diversity studies using 454 pyrosequencing (unpublished data). This resulted in a total of 245,423 high quality 16S rRNA sequences with each sample producing an average of 13,062 sequences (range = 7913–16476, SD = 3096). After PyNAST alignment and 100 % sequence identity clustering analysis, 11,790 representative sequences were obtained. RDP combined with the BLAST homology sequence alignment cluster method were then used to identify the sequences to phylum and genus level. The sequences were distributed amongst seven phyla (Fig. 2.3), four of which were the bacterial phyla *Firmicutes*, *Proteobacteria, Bacteroides* and *Actinobacteria* which together accounted for 99 % of the sequences. A further two of the phyla were also bacteria, TM7 and *Verrucomicrobia*, but they were represented by only 0.01 and 0.007 % of the total sequences, respectively.

All the sequences were also identified to genus level (Fig. 2.4). There were 11 genera represented in these samples (at a level of at least 0.03 % of sequences/sample), of which XX were LAB and, of those, *Lactobacillus* was regarded as the most abundant. In some five samples 99 % of sequences could be attributed to

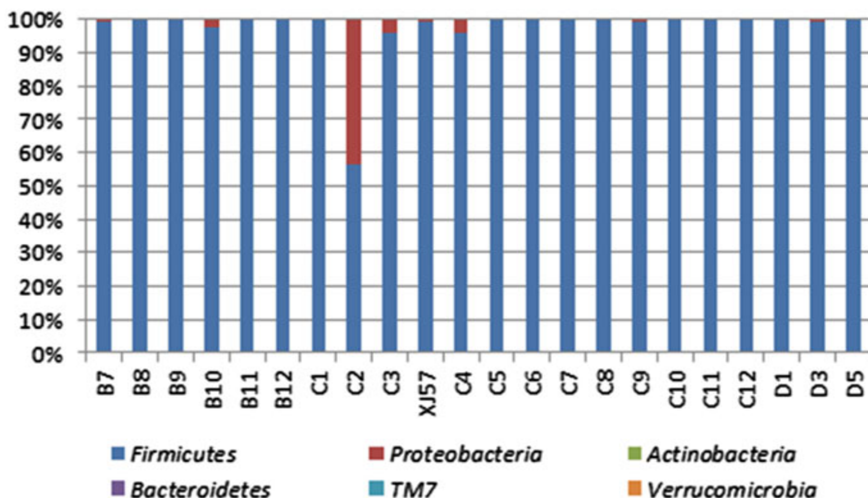


Fig. 2.3 The proportion of 16S rRNA sequences from samples of traditionally fermented cow's milk attributable to phyla of microorganisms

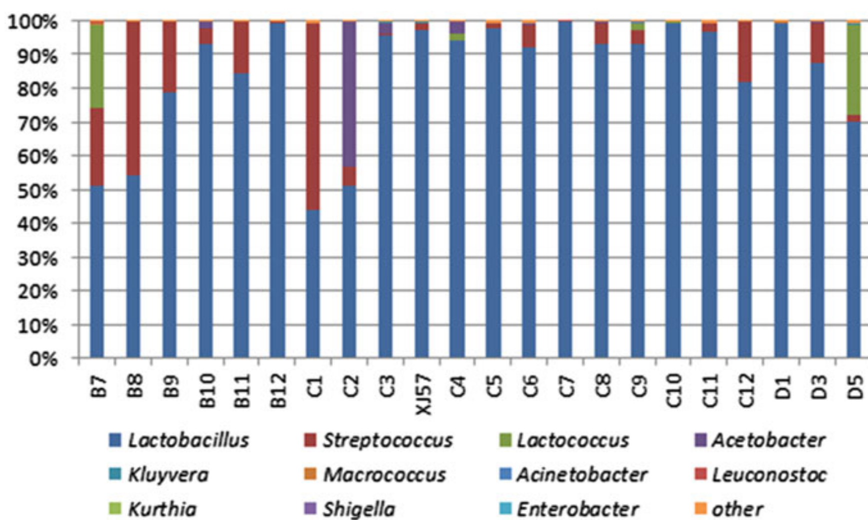


Fig. 2.4 The proportion of 16S rRNA sequences from samples of traditionally fermented bovine milk attributable to genera of microorganisms

Lactobacillus. The next most abundant genus was *Streptococcus* which, at its maximum in sample one sample represented 55 % of sequences. The highest proportion of sequences attributable to *Lactococcus* was 26.85 and 24.77 % in two samples, respectively.

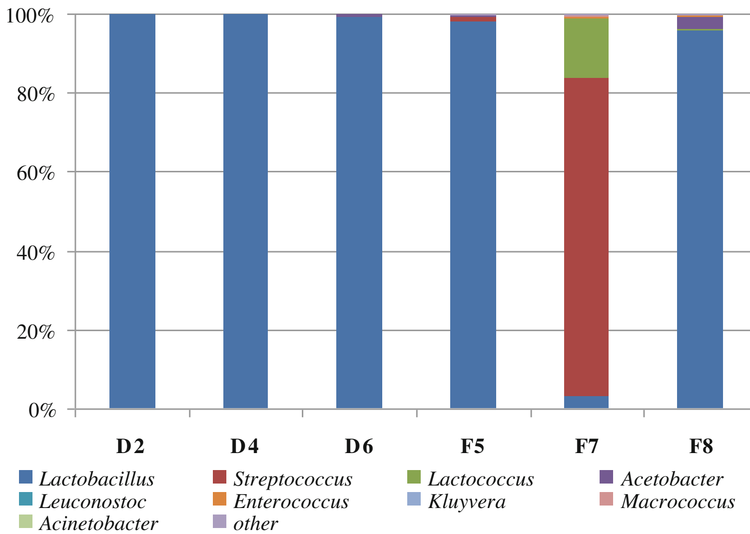


Fig. 2.5 Bacterial relative content (16S rRNA sequence proportion) in traditional koumiss based on the level of genera

Microbial Diversity of Traditional Kumiss

Microbial diversity in traditional koumiss were also studied by using the pyrosequencing technology (these data has not been published). For example, six koumiss samples (three samples from Xinjiang of China, three samples from Russia) collected from the Xinjiang Uygur Autonomous Region and Kalmuckia region of Russia. All the sequences were identified to genus level shown in Fig. 2.5, which represented the relative content of the bacteria on the genus level (greater than 0.3 %) in these samples. There were 9 genus presented these samples, of which *Lactobacillus* was regarded as the most abundant bacteria. Its content got to more than 98 %, especially in four of samples. Followed by the *Streptococcus* genus, the maximum content of these bacteria got to 81 % in one sample. Moreover, the highest content of *Lactococcus* was 14.83 % in one of samples. The other genus resented these samples were less than 10 %. All the sequences were identified to 11 phyla, Firmicuters are the predominated microflora in most samples (Fig 2.6).

Microbial Diversity in Traditional Kurut

Samples of kurut (traditionally fermented yak's milk) were collected from the Tibet Autonomous Region, China (n = 8 samples from Geda County, and 8 from Ningzhong County) and subjected to microbial diversity studies using 454 pyrosequencing analysis (unpublished data). The samples for sequencing were allocated to the different regions of two County. This resulted in 112,173 high

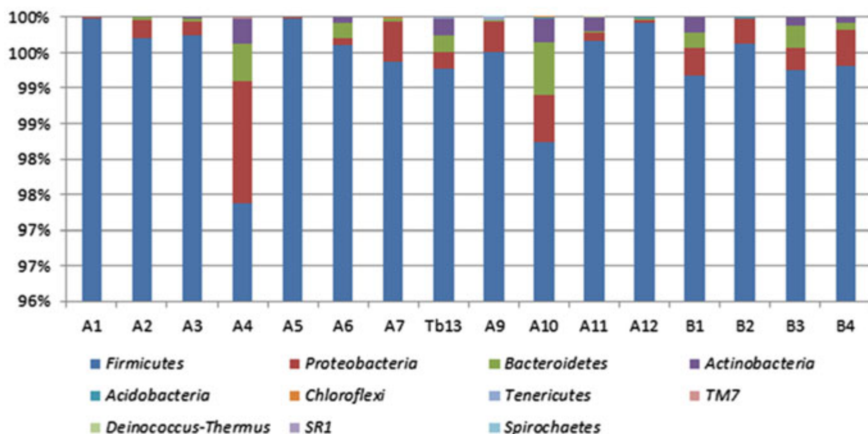


Fig. 2.6 The proportion of 16S rRNA sequences from samples of kurut attributable to phyla of microorganisms

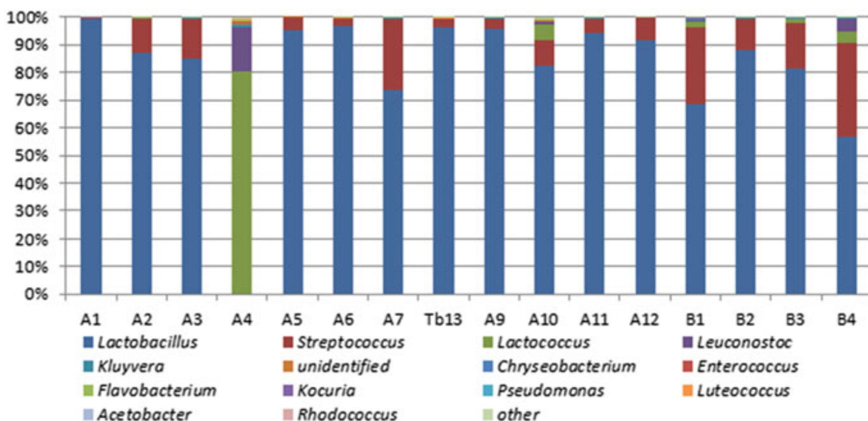


Fig. 2.7 The proportion of 16S rRNA sequences from samples of kurut attributable to genera of microorganisms

quality 16S rRNA sequences with each sample producing an average of 13062 sequences (range = 6274–7765, SD = 482). Following PyNAST alignment and 100 % sequence identity clustering analysis, 6530 representative sequences were obtained. RDP combined with the BLAST homology sequence alignment cluster method were then used to identify the sequences to phylum and genus level. The sequences were distributed amongst 11 phyla (Fig. 2.7) of which four were the bacterial phyla *Firmicutes*, *Proteobacteria, Bacteroides* and *Actinobacteria* which together accounted for 99 % of the sequences. The remaining seven phyla were also bacteria (*Acidobacteria*, *Chloroflexi, Tenericutes*, TM7, *Deinococcus-Thermus*, *Spirochaetes* and *SR1*) each accounting for between 0.013 and 0.015 % of the sequences.

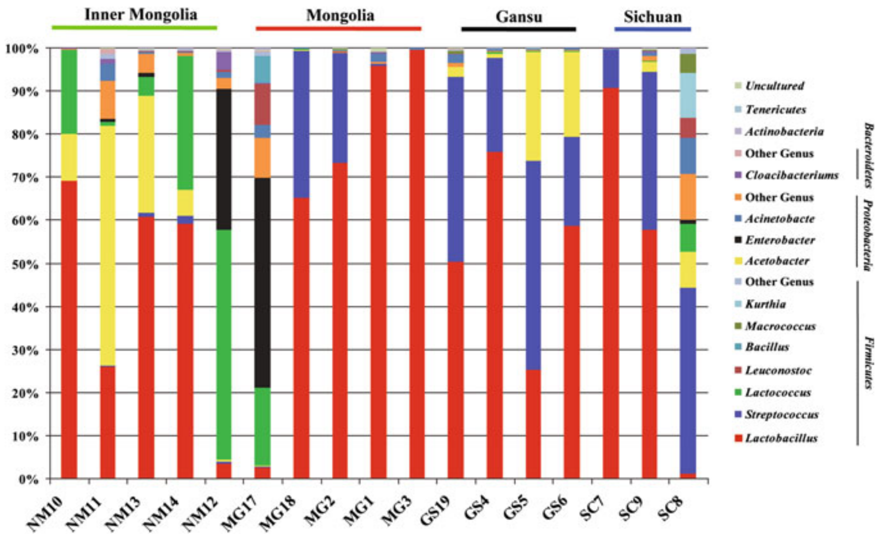


Fig. 2.8 Relative abundances (percentage of sequences) of the bacteria in naturally fermented dairy products in different regions

The sequences were also identified to genus level (Fig. 2.8). There were 13 genera identified in these samples (each at a level of at least 0.01 % of the sequences present), of which XX were LAB and, of those, *Lactobacillus* was regarded as the most abundant, representing 99 % or more of the sequences in a number of the samples. The second most abundant was *Streptococcus* genus, which was at its highest levels (33.89 % of sequences) in sample B4. In sample A4 *Lactococcus* reached its highest levels (80.20 % of sequences).

Microbial Diversity in Traditional Dairy Products in Different Places

The bacterial community diversity in fermented dairy products from Mongolia, Gansu Province, China; Sichuan Province, China and Inner Mongolia Autonomous Region, China were also examined using a metagenomic approach involving high-throughput 454 pyrosequencing (unpublished data) (Fig. 2.8). The sequences were distributed among four bacterial phyla, namely Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria. Of the four bacterial phyla, *Firmicutes* dominated in all samples from all locations. The second most dominant bacterial phyla was Proteobacteria, representing on average 17 % (±9 %) of the sequences. Bacteroidetes (0.53 % ± 0.59 %), Actinobacteria (0.11 % ± 0.05 %) and unclassified bacteria (0.11 % ± 0.07 %) were at low levels in all samples. At the genus level, a total of 95 bacterial genera were identified from the four phyla, of which *Lactobacillus* was the most abundant. At the depth of analysis carried out, 14 genera were found to be common in all the naturally fermented dairy product samples

from the four different sampling locations (Mongolia, Gansu Province, Sichuan Province and Inner Mongolia Autonomous Region). In addition to *Lactobacillus* (67.22, 52.51, 49.91 and 43.70 % for each region respectively) these were *Streptococcus* (12.07, 33.55, 29.51 and 0.71 %), *Lactococcus* (3.66, 0.17, 2.26 and 21.86 %), *Leuconostoc* (1.92, 0.02, 1.60 and 0.16 %) species of LAB.

Simultaneously, *Acinetobacter* (1.13, 0.75, 3.09 and 1.31 %), *Enhydrobacter* (0.15, 0.16, 0.42 and 0.37 %), *Cloacibacterium* (0.15, 0.09, 0.18 and 1.09 %), *Acetobacter* (0.10, 12.09, 3.57 and 20.06 %), *Macrococcus* (0.05, 0.17, 1.52 and 0.02 %), *Chryseobacterium* (0.05, 0.03, 0.02 and 0.12 %), *Delftia* (0.03, 0.01, 0.03 and 0.12 %), *Escherichia* (0.02, 0.05, 0.06 and 0.04 %), *Rhizobium* (0.02, 0.01, 0.16 and 0.09 %) and *Roseomonas* (0.02, 0.01, 0.03 and 0.06 %) were identified.

2.3.2.2 LAB Diversity in Traditionally Fermented Dairy Products as Determined by Other Molecular Techniques, Particularly Denaturing Gradient Gel Electrophoresis

In addition to metagenomic sequence analysis, there are other molecular techniques that allow culture-independent identification and monitoring of microbial diversity. Most of these techniques are based on polymerase chain reaction (PCR) amplification and detection of the predominant nucleic acids present in a sample (Yang et al. 2001). Compared to conventional methods these molecular methods are generally faster, more specific, more sensitive and more accurate, allowing a precise study of microbial populations and their diversity in given ecosystems. These have been useful to monitor changes in microbial communities.

Culture-independent studies based on molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (Ercolini et al. 2004; Lafarge et al. 2004), temporal temperature gradient electrophoresis (TTGE) (Lafarge et al. 2004; Ogier et al. 2002), single strand conformation polymorphism (SSCP) (Duthoit et al. 2003) have been used to characterise bacterial diversity in raw milk and other dairy products and fermented food (Table 2.14). These culture-independent methods can not only fully characterise the primary LAB from milk such as *Lactobacillus, Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Weissella and Pediococcus*, but can also detect the large spectrum of other microbes that occur less frequently or cannot be detected by culture-dependent methods (Table 2.15).

2.4 Review of Biodiversity of Lactic Acid Bacteria in Fermented Foods

2.4.1 Summary

Globally, in the past century, many microorganisms have been isolated and characterised from traditionally fermented food. LAB have received considerable

attention from the pharmaceutical and /or food fermentation industries because of their biotechnology potential. LAB have an essential role in the dairy industry because of the high levels of human consumption of several important fermented food products, mainly cheese and fermented milks. The most important LAB belong to the nonpathogenic genera *Lactococcus, Lactobacillus, Leuconostoc, Oenococcus* and *Streptococcus* which have been used traditionally in the food industry. Numerous stains of these genera have been isolated and identified from dairy products, grain products, meat and fish products, beer, wine, fruit and fruit juices, pickled vegetables, mash, sauerkraut, silage, sourdough, etc. from various regions including Europe, Asia, Africa and Latin America. In this section, we review and list the predominant species of LAB from different fermented food.

2.4.2 History of LAB in Fermented Food

The definition of 'fermentation' is 'a process in which chemical changes are brought about in an organic substrate through the action of enzymes produced by microorganisms' (Prescott and Dunn 1957; Psoni et al. 2003). Although it is extremely difficult to pinpoint the precise beginnings of human awareness of the presence and role of microorganisms in foods, the available evidence indicates that this knowledge preceded the establishment of bacteriology or microbiology as fields within science. The exploitation of fermentation in human society has a long history. The first evidence of beer manufacture has been traced to 7000 BC in ancient Babylon (Pederson 1971). At around 3000 BC, the Sumerians are believed to have been the first great livestock breeders and dairymen and were among the first to make butter (Tamime 2002). Milk, butter and cheese were also used by the Egyptians as early as 3000 BC (Jay et al. 1996). In China, koumiss has a long history of use as a popular drink among traditional nomadic people. The earliest records of koumiss appeared during the Han dynasty (202 BC–202 AD) and it attained widespread popularity during the Yuan dynasty (1271 AD–1368 AD).

Kircher (1659) first demonstrated the occurrence of bacteria in milk and this was confirmed by Bondeau (1846) two centuries later. Leeuwenhoek (1680) was the first to observed yeast cells in milk but it was Pasteur (1857) who was the first scientist to show that souring of milk was caused by the growth of microorganisms within it. By the turn of the twentieth century, the term lactic acid bacterium or LAB was used to mean 'milk-souring organisms'. Significantly, the first pure culture of a bacterium was *Bacterium lactis* (likely to be what we now call *Lc. lactis*) (Lister 1873) and Metchnikoff et al. (1907) isolated and named a bacterium from, *Lb. delbrueckii* subsp. *bulgaricus*.

Table 2.15 Species composition of LAB and other microorganism from fermented dairy products as determined by culture-dependent and culture-independent molecular fingerprinting approaches used simultaneously against the same samples

Country/region	Dairy Product	Microorganisms detected by culture-dependent methods	Microorganisms detected by culture-independent molecular fingerprinting methods	Culture-independent method used	References
Italy	Cow's milk cheese	*E. sulfurans/faecalis* *E. hiraea* *Lb. fermenteum/plantaraum/casei* *Lc. lactis* *Leuc. mesenteroides* *P. acidilactici* *S. thermophilus*	*Enterococcus sulfurans/faecalis* *Lb. fermentum/plantarum/casei/delbrueckii* subsp. *bulgarcus* *Lc. lactis* *Leuconostoc mestenteroides* *Pediococcus acidilactici* *Streptococcus thermophilus/ bovis*	DGGE	Randazzo et al. (2002)
North East of Italy	Cow's milk	A further other microorganism species that were not LAB' *Candida catenulata/pararugosa/parapsilosis/zeylanoides/pseudointermedia/rugosa* *Cryptococcus curvatus* *Kluyveromyces marxianus/lactis* *Pichia guilliermondii* *Saccharomyces cerevisiae* *Trichosporon mucoides*	*Candida pseudorugosa/kefyr/pseudointermedia/humilis/rugosa* *Galactomyces* spp. *Kluyveromyces marxianus/lactis* *Macrococcus caseolyticus* *Saccharomyces bayanus/cerevisiae*	DGGE	Cocolin et al. (2002)
Germany	Cow's milk cheese	*Lb. paracasei* subsp. *paracasei/ delbrueckii* subsp. *bulgaricus/delbrueckii* subsp. *lactis/casei*	*Lactobacillus paracasei* subsp. *paracasei/plantarum*	TTGE	Henri-Dubernet et al. (2008)

(continued)

Table 2.15 (continued)

Country/ region	Dairy Product	Microorganisms detected by culture-dependent methods	Microorganisms detected by culture-independent molecular fingerprinting methods	Culture-independent method used	References
France	Cow's milk cheese	*Lactobacillus curvatus* subsp. *curvatus* *Lactococcus lactis* subsp. *lactis* *S. thermophilus* A further other microorganism species that were not LAB' *Brevibacterium linens* *Brachybacterium species* *Carnobacterium maltaromaticum* *Corynebacterium casei* *Marine bacterium* *Marinolactibacillus psychrotolerans* *Microbacterium gubbeenense*	*Lactobacillus curvatus* subsp. *curvatus* *Lactococcus lactis* subsp. *lactis/cremoris* *Streptococcus thermophilus* *Brevibacterium linens* *Brachybacterium species* *Carnobacterium maltaromaticum* *Corynebacterium casei* *Marine bacterium* *Marinolactibacillus psychrotolerans* *Microbacterium gubbeenense* *Pseudoalteromonas species* Uncultured Flavobacterium	SSCP	Feurer et al. (2004)
France	Cow's milk cheese	*Kluyveromyces lactis/marxianus* *Kluyveromyces* *Candida* *zeylanoides/parapsilosis/silvae/intermedia/tropicalis/rugosa*	*Kluyveromyces lactis/marxianus* *Kluyveromyces* *Candida* *zeylanoides/parapsilosis/silvae/intermedia*	SSCP	Callon et al. (2006)
France	Cow's milk cheese	*Enterococcus faecalis* *Lactobacillus casei* *Lactococcus lactis* *Lactococcus garvieae* *S. thermophilus/dysgalactiae* Plus a further 29 species that were not LAB	*E. faecalis* *Lb. casei/kefiranofaciens* *Lc. lactis* *S. dysgalactiae/thermophilus* Plus a further 23 species that were not LAB	SSCP	Delbes et al. (2007)

(continued)

Table 2.15 (continued)

Country/region	Dairy Product	Microorganisms detected by culture-dependent methods	Microorganisms detected by culture-independent molecular fingerprinting methods	Culture-independent method used	References
France	Goat's milk	*E. faecalis*	*E. faecalis/saccharominimus*	SSCP	Callon et al. (2007)
		E. saccharominimus	*Lb. lactis/garvieae/casei*		
		Lb. casei	*Leuc. mesenteroides*		
		Lc. lactis/garvieae	*S. mitis*		
		Leuc. mesenteroides	Plus a further 34 species that were not LAB		
		S. mitis			
		Plus a further 35 species that were not LAB			
Belgium	Cow's mik cheese	*Lb. paracasei/plantarum/brevis/curvatus/rhamnosus/perolens*	*E. faecalis*	DGGE	Van Hoorde et al. (2008)
		Lc. lactis subsp. *lactis*	*Lb. paracasei/plantarum/brevis/curvatus/rhamnosus/parabuchneri/gallinarum*		
		P. pentosaceusa	*Lc. lactis* subsp. *lactis*		
		S. salivarius	*P. pentosaceus*		
		Weissella paramesenteroides			
Serbia	Goat milk cheese	*E. faecalis*	*Lb. paracasei* subsp. *paracasei*	DGGE	Nikolic et al. (2008)
		Lb. paracasei subsp. *paracasei*	*Lc. lactis* subsp. *lactis*		
		Lc. lactis subsp. *lactis*	*Leuc. mesenteroides*		
	Goat's milk cheese	*Lb. casei/buchneria/paracasei* subsp. *paracasei/tolerans/rhamnosus*	*Lb. casei/delbrueckii* subsp.	DGGE	Gala et al. (2008)
		P. acidilacticia	*lactis/parabuchneri/fermentum/rhamnosus*		
Italy	Cow's milk cheese	*E. faecium*	*Lb. plantarum/kefiranofaciens*	DGGE	Dolci et al. (2008)
		E. faecalis	*Lc. lactis* subsp. *lactis*/subsp. *cremoris*		
		Lb. plantarum/paracasei/casei/coryneformis subsp. *torquens/delbrueckii* subsp. *lactis*	*Streptococcus agalactiae*		
		Lc. lactis subsp. *lactis*/subsp. *cremoris*	Other microorganism species that were not LAB		
			Macrococcus caseolyticus		

(continued)

Table 2.15 (continued)

Country/ region	Dairy Product	Microorganisms detected by culture-dependent methods	Microorganisms detected by culture-independent molecular fingerprinting methods	Culture-independent method used	References
France	Cow's milk cheese	*Lb. paracasei/helveticus/rhamnosus/ parabuchneri/fermentum/perolens/ acidophilus/brevis/kefiri/delbrueckii* subsp. *bulgaricus/delbrueckii* subsp. *lactis*	*Lb. paracasei/plantarum/acidophilus/ rhamnosus*	TTGE	Henri-Dubernet et al. (2008)
Italy	Cow's milk cheese	*Lb. helveticus/delbrueckii* subsp.	*Lb. helveticus/delbrueckii*	DGGE	Aponte et al. (2008)
		E. faecalis/durans/faeciuma	subsp. *lactis*		
		Lb. subsp. *bulgaricus/delbrueckii*	*Lc. lactis*		
		subsp. *delbrueckii/delbrueckii* subsp. *indicus/ fermentum*	*S.thermophilus*		
		Lb. paracasei/rhamnosus	*Weisella species*		
		Lc. Garvieae/lactis			
		Leuc. mesenteroides subsp. *lactisa*	Other microorganism species that were not LAB		
		P. acidilacticia	*Macrococcus caseolyticus*		
		S. thermophilusa/parauberis/haemolyticus/ croceolyticus/warneri/pasteuria	*Morexella osloensis*		
		Other microorganism species that were not LAB	*Rahnella species*		
		Macrococcus caseolyticusa	*Aeromonas simiae*		
Spanish	Goat and sheep's milk cheese	*E. devriesei/faecium*	*E. species*	TTGE	Abriouel et al. (2008)
		Lb.s paracasei/plantarum/brevis	*Lactobacillus plantarum/brevis/*		
		Lc. lactis	*acidophilus/paracasei*		
		Leuc.c mesenteroides/pseudomesenteroides	*Lc. Lactis*		
		P. urinaequi			
		Other microorganism species that were not LAB	Other microorganism species that were not LAB		
		Hafnia alvei			
		Escherichia coli	*Escherichia coli*		
		Obesumbacterium proteus	*Nitrogen-fixing bacterium*		
		Shigella flexneri			

(continued)

Table 2.15 (continued)

Country/region	Dairy Product	Microorganisms detected by culture-dependent methods	Microorganisms detected by culture-independent molecular fingerprinting methods	Culture-independent method used	References
Italy	Sheep and goat's milk cheese	*Lb. planatrum/brevis/coryneformis/ paraplantarum* Other microorganism species that were not LAB *Kluyveromyces lactis* *Pichia membranifaciens/fermentans* *Candida krisii/zeylanoides*	*Lb. delbrueckii* subsp. *bulgaricus /plantarum/helveticus/* *suntoryeus/gallinarum* *Lc. lactis* *S. thermophilus* Other microorganism species that were not LAB *Kluyveromyces lactis* *Pichia membranifaciens/fermentans* *Candida zeylanoides*	DGGE	Rantsiou et al. (2008)
Italy	Goat's milk cheese	*E. faecium/durans/faecalis/* *malodoratus* *Lb. curvatus/plantarum* *Lactococcus lactis* subsp. *lactis* *Streptococcus thermophilus*	*Lb. plantarum* *E. faecium/durans/faecalis/* *malodoratus* *Lc. lactis* subsp. *Lactis* *Streptococcus species* Other microorganism species that were not LAB *Staphylococcus haemolyticus* *Escherichia coli* *Clostridium bifermetans/Eubacterium* *tenue* Unidentified bands	DGGE	Serhan et al. (2009)
Italy	Sheep's milk cheese	*E. faecalis* *Lb. rhamnosus/brevis* *Leuc. mesenteroides* *Lc.lactis* *S. thermophilus*	*E. faecalis* *Lb. rhamnosus/brevis/* *plantarum/pentosus/fermentum/* *buchneri/delbrueckii* *Leuc. mesenteroides* *Lc. lactis* *S. thermophilus*	DGGE	Randazzo et al. (2009)

(continued)

Table 2.15 (continued)

Country/ region	Dairy Product	Microorganisms detected by culture-dependent methods	Microorganisms detected by culture-independent molecular fingerprinting methods	Culture-independent method used	References
Lebanon	Cow's milk cheese	*Lb. plantarum*	*E. faecium*	DGGE	Alegria et al. (2009)
		Lactococcus lactis/garvieae	*Lb. plantarum/casei/paracasei*		
		Leuc. mesenteroides	*Lc. lactis/garvieae*		
		Other microorganism species that were not LAB	*S. thermophilus/parauberis/uberis/iniae*		
		Staphylococcus saprophyticus/pasteuri	Other microorganism species that were not LAB		
		Klebsiella sp.	*Corynebacterium variable*		
		Escherichia coli	*Macrococcus caseolyticus*		
		Micrococcus luteus	*Geotrichum candidum*		
		Corynebacterium variable	*Kluyveromyces sp.*		
		Flavobacterium species	*Saccharomyces sp.*		
		Microbacterium oxydans	*Trichosporon gracile*		
		Musa acuminata			
Italy	Goat's milk cheese	*Lb. pentosus/brevis/plantarum*	*Lc. lactis* subsp. *lactis*	DGGE	Alessandria et al. (2010)
		Lc. subsp. *lactis/garvieae*	*Lb. helveticus*		
		E. faecium/casseliflavus/faecalis/italicus/ durans	*Leuc. pseudomesenteroides*		
		Leuc. citreum/mesenteroides			
		P. pentosaceus	Other microorganism species that were not LAB		
		Weissella paramesenteroides	*Candida pararugosa*		
		Aerobasidium pullulans	*Rhizomucor miehei*		
		Candida paraugosa/zeylanoides/parapsilosis	*Alternaria alternata*		

(continued)

Table 2.15 (continued)

Country/ region	Dairy Product	Microorganisms detected by culture-dependent methods	Microorganisms detected by culture-independent molecular fingerprinting methods	Culture-independent method used	References
Spain	Cow's milk cheese	*Lactococcus lactis* subsp. *lactis/*	*Lb. casei/helveticus*	DGGE	Dolci et al. (2010)
		subsp. *cremoris*	*Lc. lactis* subsp. *lactis/*subsp.*cremoris*		
		Lb. casei/plantarum/coryneformis	*S. agalactiae*		
		subsp. *torquens/acidipiscisa*	Other microorganism species that were not LAB		
		Streptococcus thermophilus/agalactiaea	*Spingomonas* sp.		
Turkey	Fermented dry sausage	*Lb. Alimentarius/brevis/farciminis/sakei/ curvatus/plantarum*	*Lb. brevis/curvatus/farciminis/sakei*	16S rRNA V_3 Sequencing	Kesmena et al. (2012)
		W. halotolerans	*Lc. piscium*	Rep-PCR	
			Leuc. mesenteroides/citreum	DGGE	
			W. viridescens		
Spain	Rioja wine	*Lb.mali/Lb.plantarum*	*Lb. Buchneri/plantarum*	DGGE	González-Arenzana et al. (2013)
		Leuc. fallax/mesenteroides	*Lc.lactis*		
		O. oeni	*Leuc. mesenteroides*		
		P. pentosaceus	*O. kitaharae/oeni*		
			P. parvulus/pentosaceus		

2.4.3 LAB in Dairy Products

Milk is one of the most important components of human diet from infancy to old age (Elmagli and El-Zubeir 2006) and it can be fermented by LAB and yeast to produce other products that vary in their taste, constituents and shapes. It is likely that the origin of dairy products was the Middle East and the Balkans (Tamime 2002). Today, various types of fermented dairy products have been developed in all parts of the world including the Middle East, South Africa, Asia and Western Europe. There were approximately 400 generic names applied to traditional and industrialised products varying only in the kind of milk fermented (Kurmann et al. 1992). In recent years, with the advancement of study on dairy products, more and more species of LAB have been isolated and characterised from previously unknown natural fermented dairy products in remote rural areas. The most important varieties of dairy products worldwide, and the LAB within them are, alphabetically, as follows:

Amasi is a well-known sour cow milk drunk traditionally by the Zulu people of South Africa. It is produced in clay pots and gourds which are used repeatedly to ensure that spontaneous fermentation of the raw milk introduced to them occurs naturally at ambient temperature of ±5 °C (Keller and Jordan 1990; Gadaga et al. 2001). Traditional preparation of amasi is similar in other countries where raw milk is poured into calabashes made from gourds or into stone jars. It is then left to ferment for several days. Amasi is now available in commercial outlets in South Africa. There were seven species of LAB isolated from Amasi by different researchers (Table 2.16). In a subsequent study by Feresu and Muzondo (1990), the predominant LAB isolated from amasi were identified as *Lb. helveticus*, *Lb. plantarum*, *Lb. delbrueckii* subsp. *lactis*, *Lb. paracasei* subsp. *paracasei* and *Lb. paracasei* subsp. *pseudoplantarum* (Feresu and Muzondo 1990).

Biruni is a cheese-like product made from XX's milk that is limited to the Nuba Mountains area of Sudan although it has recently spread into more areas inhabited by pastoralists who have named it laban-gadim (aged milk). It is similar to mish which is also produced in Sudan. Biruni has probably one of the longest shelf lives of any fermented milk product as it can be stored for at least 1 year and maybe as long as 10 years (Salih et al. 2011). Information on the product is scanty, however, because it comes from the region of the Nuba Mountains which is in the vicinity of the war-torn township of Dialing (Abdelgadira et al. 1998).

Branza dulce, **telemea and urda** are common names for Romanian artisan dairy products (Zamfir et al. 2006). The cows' or goats' or buffalos' milk is left overnight to ferment in a warm place (for instance, next to the oven in the farm house). In some cases the milk is first boiled for several minutes. On the second day, the upper layer is collected as fermented sour cream and the rest is used as fermented milk (sour milk) or for cheese production. For the latter, the sour milk is warmed up and rennet is added. Alternatively, rennet can be added to the milk before fermentation. The whey is then removed from the sour milk and the cheese curd is collected in a special cloth, pressed and drained. This so-called branza

Table 2.16 Species of LAB and yeast found in traditional fermented dairy products from around the world

Country/Regions	Name of dairy product	Type of products	Raw material	Species of LAB present	Species of yeast present	Counts of microorganisms as CFU/g	References
South Africa	Amasi	Fermented milk	Cow's milk	3, 4, 9, 22, 27, 35, 38, 56	B, C, D, E	1.2×10^5 to 1.97×10^6	Salih et al. (2011) Zamfir et al. (2006) Chelule et al. (2010)
Kenya	Sethemi	Fermented milk	Milk		B, D, F, G	1.0 x 10^6	Kebede et al. (2007)
	Kule naoto	Fermented milk	Milk	4, 9, 35, 38, 42, 50, 55		1.0×10^7 to 1×10^9	Mathara et al. (2008)
Sudan	Gibna bayda	Cheese		19, 13, 66		ND	Ahmed et al. (2009)
	Gibna mudaffara	White pickled cheese			ND	ND	
	Gariss	Fermented milk	Camel's milk	3 or 4, 9, 11, 13, 24, 38, 35,51, 52		ND	Sulieman et al. (2006), Abdel Moneim et al. (2006), Hassan et al. (2008), Ashmaig et al. (2009)
	Rob (Roub or Robe) also named kit, laben-rayeb	Fermented milk	Cow's, or sheep and goat's milk	13, 19, 24, 27, 37, 38, 42, 51	A, B, C	1×10^7 to 1×10^8 (for species of *Lactococcus* and *Lactobacillus*) 1×10^6 to 1×10^7 (for yeasts)	Saeed (1981), El Mardi (1988)
	Zabadi	Fermented milk	Cow's milk	19, 65	H	ND	Abdelgadira et al. (1998)
Ghana	Nunu	Yogurt-like product	Cow's milk	*Lactobacillus* spp. *Leuconostoc* spp. *Enterococcus* spp. *Streptococcus* spp.	B, C, F	1×10^4 to 1×10^8 (for LAB) -1×10^6 (for yeasts)	Akabanda et al. (2010)
Netherlands	Gouda	Dutch-type cheese	Cow's, sheep or goat's milk	3, 11, 18, 34, 35, 38, 42, 54, 62, 67		1×10^7 to 1×10^8	Van Hoorde et al. (2008), Koenraad et al. (2008)
Italy	Parmigiano Reggiano	Cheese	Raw and partly skimmed cow's milk	13, 19, 22, 24, 34, 35, 42,			Gala et al. (2008)

(continued)

Table 2.16 (continued)

Country/ Regions	Name of dairy product	Type of products	Raw material	Species of LAB present	Species of yeast present	Counts of microorganisms as CFU/g	References
Morocco	Jben	Soft white cheese	Milk	1, 2, 3, 4, 11, 12, 38, (Park et al. 2006). Dewan S, Tamang 42, 49, 50, 52, 53, 55, 57, 65		1×10^8 to 1×10^9	Ouadghiri et al. (2005)
Greek	Batzos	Cheese	Goat's milk	18, 38, 35, 37, 44		ND	Psoni et al. (2003)
France	Emmental	Comté Camembert	Cow's milk	9, 13, 19, 22, 27, 35, 38		ND	Gagnaireet al. (2001), Bouton et al. (2002)
Caucasus	Kefir	Tart and mildly alcoholic effervescent fermented milk	Milk	11, 19, 27, 29, 32, 51, 65	B, D, E, F,	ND	Simova et al. (2002), Chen et al. (2008)
China	Qula	Yellow or white cheese	Yak's milk	3, 4, 38, 56, 57, 62		1×10^3 to 1×10^7 (for LAB) 0 to 1×10^5 for mould 1×10^3 to 1×10^8 for yeasts	Bao et al. (2012a), (b)
	Kurut	Fermented yak's milk	Yak's milk	2, 3, 4, 11, 13, 19, 24, 27, 32, 38, 50, 51, 52, 53, 54, 56, 65, 68		–	Sun et al. (2010), Bao et al. (2012a, b)
	Dairy fan	Cheese-like product	Goat's milk	2, 24, 25, 27, 28, 30, 31, 32, 38, 51, 53, 54		–	Liu et al. (2009)
	Milk cake	Cheese-like product	Cow's or goat's milk	2, 19, 27,49,50,51, 65		8.32×10^{10} to 10.16×10^{10} (for LAB) 1.6×10^{10} to 6.15×10^{10} (for yeasts)	Bao et al. (2011)
	Chige						
Romania	Branza dulce/ Urda/ Telemea	Sweet cheese	Goat's and buffalo's milk	2, 3, 4, 11, 18, 31, 35, 49, 51, 52, 55, 57, 63, 65, 71		1×10^7 to 9×10^9 including commercial products	Zamfir et al. (2006)

(continued)

Table 2.16 (continued)

Country/ Regions	Name of dairy product	Type of products	Raw material	Species of LAB present	Species of yeast present	Counts of microorganisms as CFU/g	References
Egypt	Kishk						
	Laban zeer	Sour buttermilk	Milk				
	Domiati cheese (Gbnah Beeda)	Soft white pickled cheese	Cow's or buffalo's milk	3, 9, 11, 13, 25, 38, 49, 50, 51, 52, 53, 55, 65		7.1 to 7.6×10^{10}	El-Baradei et al. (2007)
Senegal	M'Bannick	Kefir-like beverage					
Czech Repubic	Zincica	Salted kefir-like beverage					
India	Dahi	Curd milk or curd rice	Cow's milk or rice	10, 50, 51		1×10^7 to 1×10^8	Dewan and Tamang (2007)
	Mohi	Buttermilk beverage	Milk	10, 50, 51		1×10^7 to 1×10^8	
	Chhutpi	Cottage cheese-like product	Fermented cow's milk	4, 10, 28, 32, 35, 38		1×10^7 to 1×10^8	
	Somar	Soft paste with bitter taste	Cow's milk	35		1×10^7 to 1×10^8	Dewan and Tamang (2007)
	Philu	Cream-like milk product	Cow's or yak's milk	35		1×10^7 to 1×10^8	
	Shyow	Thick-gel curd	Yak's milk	35		1×10^7 to 1×10^8	

(continued)

Table 2.16 (continued)

Country/ Regions	Name of dairy product	Type of products	Raw material	Species of LAB present	Species of yeast present	Counts of microorganisms as CFU/g	References
Italy	Montasio cheese	Semi-hard cheese	Cow's milk				
	Mozzarella cheese		Cow's or buffalo's milk	3, 51, 54, 55, 63, 65		1×10^5 to 1×10^7	Morea et al. (1999)
	Parmigiano Reggiano	Cheese	Cow's milk	19, 22, 27, 42			Coppola et al. (2001)

Note 1. *E. casseliflavus*, 2. *E. durans*, 3. *E. faecalis*. 4. *E. faecium*, 5. *E. gallinarum*, 6. *E. italicus*, 7. *E. thailandicus*, 8. *Lb. acetotolerans*, 9. *Lb. acidophilus*, 10. *Lb. alimentarius*, 11. *Lb. brevis*, 12. *Lb. buchneri*, 13. *Lb. casei*, 14. *Lb. confuses*, 15. *Lb. coryniformis* subsp. *torquens*, 16. *Lb. crispatus*, 17. *Lb. crustorum*, 18. *Lb. curvatus*, 19. *Lb. delbrueckii* subsp. *bulgaricus*, 20. *Lb. delbrueckii* subsp. *delbrueckii*, 21. *Lb. delbrueckii* subsp. *indicus*, 22. *Lb. delbrueckii* subsp. *lactis*, 23. *Lb. diolivorans*, 24. *Lb. fermentum*, 25. *Lb. graminis*, 26. *Lb. hamsteri*, 27. *Lb. helveticus*, 28. *Lb. hilgardii*, 29. *Lb. kefiranofaciens* subsp. *kefiranofaciens*, 30. *Lb. kefiranofaciens* subsp. *kefirgranum*, 31. *Lb. kefirgranum*, 32. *Lb. kefiri*, 33. *Lb. mindensis*, 34. *Lb. parabuchneri*, 35. *Lb. paracasei*, 36. *Lb. paralimentarius*, 37. *Lb. pentosus*, 38. *Lb. plantarum*; 39. *Lb. pontis*, 40. *Lb. rapi*, 41. *Lb. reuteri*, 42. *Lb. rhamnosus*, 43. *Lb. rossia*, 44. *Lb. sakei*, 45. *Lb. sanfrancisco*, 46. *Lb. spicheri*, 47. *Lb. sunkii*, 48. *Lb. uvarum*, 49. *Lc. garvieae*, 50. *Lc. lactis* subsp. *cremoris*, 51. *Lc. lactis* subsp. *lactis*, 52. *Lc. raffinolactis*, 53. *Leuc. citreum*, 54. *Leuc. lactis*, 55. *Leuc. Mesenteroides*, 56. *Leuc. mesenteroides* subsp. *mesenteroides*, 57. *Leuc. pseudomesenteroides*, 58. *P. acidilactici*, 59. *P. damnosus*, 60. *P. ethanolidurans*, 61. *P. parvulus*, 62. *P. pentosaceus*, 63. *S. bovis*, 64 *S. parauberis*, 65. *S. thermophilus*, 66. *S. mutans*, 67. *S. salivarius*, 68. *W. cibaria*, 69. *W. confuse*, 70. *V. carniphilus*; 71. *W. viridescens*, *W. paramesenteroides*

A. *Corynebacteria pseudodiptherium*, B. *Saccharomyces cerevisiae*, C. *Candida kefyr*, D. *Cadida lusitaniae*, E. *Candida colliculosa,* F. *Kluyveromyces maxianus,* G. *Debaryomyces hansenii*, H. *Candida krusei*

dulce (sweet cheese) can be consumed immediately. Alternatively, a salted cheese called telemea can be manufactured. This can be kept for several months in a mixture of water, whey and salt. Also, a soft whey cheese, called urda, can be prepared by warming up the whey to 80–90 °C, collecting the floating particles and drying them. LAB composition in these products was described by Zamfir et al. (2006) and included 15 species. The most frequent LAB found in Romanian raw milk and fermented dairy products (fermented milks, sour cream, cheese) was *Lc. lactis*.

Chhurpi is a cottage cheese-like fermented cows' or yaks' milk product from the Indian state of Sikkim and Nepal. It has a rubbery texture with a slightly sour taste. This traditional fermented dairy product is lesser known as dahi mohi, somar, philu and shyow because they are mainly restricted to the unorganised sector as well as individual households. During production, milk is boiled for about 15 minutes until a soft, white mass is formed which is sieved out and placed in a muslin cloth and allowed to drain thereby removing the remaining whey. This produces the soft variety of chhurpi which is eaten in curry or as a soup (Dewan and Tamang 2007). When chhurpi (Sherpas call it sherkam) is kept in a closed vessel for about 15 days or more, the fermented product is called somar which is a strong-flavoured, bitter but soft paste that is consumed as soup alongside cooked rice or finger millet by the Sherpas of Sikkim and Nepal. 128 isolates were identified to *Lb. bifermentans, Lb. paracasei* subsp. *pseudoplantarum*, *Lb. kefir*, *Lb. hilgardii*, *Lb. alimentarius*, *Lb. paracasei subsp. paracasei*, *Lb. plantarum*, *Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris* and *E. faecium* from these dairy product collected from different places of India, Nepal and Bhutan (Dewan and Tamang 2007).

Dairy fan is also called 'rusan' or 'ruxian' in the Chinese language because of its unique fan shape. It is a cheese-like product made from cows' milk and consumed by the Bais people, an ethnic minority from Yunnan Province and Southwest China. The chemical composition and nutritional compositon was evaluated by Liu et al. (2011). Dairy fan is produced by adding an 'acid juice' to the milk and heating it up (Zhang 2008). Liu et al. (2009) isolated 91 strains of LAB from 20 samples of dairy fan. These isolates could be attributed to 12 species or subspecies based on their physiological and biochemical characteristics, and 16S rRNA gene sequence analysis. *Lactobacillus helveticus* and *Lb. fermentum* were the predominant species in dairy fan from Yunnan.

Dahi is local Nepalese name for curd prepared from cow's milk and prepared in most rural households for direct consumption or for subsequent processing into other ethnic milk products like gheu, mohi and chhurpi (Dewan and Tamang 2007). For traditional dahi preparation, fresh cow's milk is first boiled in a vessel. After boiling, the milk is cooled to room temperature and a small quantity of previously prepared dahi is added and is left for 1–2 days in summer or for 2–4 days in winter at ambient room temperature for natural fermentation to occur. Dahi is also traditionally eaten with boiled rice or 'chewra' (beaten-rice) by Nepalese people.

Gariss is a unique Sudanese traditionally fermented camel's milk product. It is made in a semi-continuous fermentation process. The word Gariss means 'pinching' or 'stinging', denoting a high degree of sourness. Fermentation is done in two leather bags of tanned goat skin embedded in green or wet grass carried on the backs of camels. In this way, the milk is subjected to continuous shaking by the movement of the camels. Whenever part of the product is withdrawn for consumption, fresh camel's milk is added to make up the volume. This production process described by Salih et al. (2011) is similar to the procedure used for the traditional production of koumiss by the original nomadic Mongolian people in China. The LAB composition of Gariss includes more than 10 species isolated from the dairy products (Table 2.16). The most dominant species from the genus *Lactobacillus* were *Lb. paracasei* group, *Lb. fermentum*, *Lb. plantarum* and *Lb. raffinolactis.* The dominant coccoid species were *Lc. lactis, Enterococcus* spp. and *Leuconostoc* spp. (Sulieman et al. 2006; Abdel Moneim et al. 2006; Hassan et al. 2008; Ashmaig et al. 2009).

Gibna (cheese) production from different raw milk (including cow's buffalo's milk) in Sudan began in the early eighteenth century by Greek families who had migrated to Sudan. Gibna making is the major preservation method for surplus milk in rural areas. The major types of cheese made are Gibna Bayda and Gibna Mudaffara (El-Sheikh 1997; Hamid and El-Owni 2007) and they are vary in composition, texture, colour, taste and flavour, type of packaging and microbial activity during ripening (Salih 2011). Gibna bayda is unique in containing high concentrations of salt that are added to the milk before processing to prevent rapid deterioration before ripening can occur (Taormian 2010). The production process has been reviewed by Salih et al. (2011) mainly including some steps such as salting, coagulum fermentation, fitting, curd and so on. The LAB and other bacteria naturally present in the raw milk carry out the fermentation process and no starter is required (Abdel Gadir et al. 1998; El-Owniand Hanmid 2008). *Lb. delbrueckii* subsp. *bulgaricus, Lb. casei and S. mutans* was the most common microflora of the products (Ahmed 1997). Gibna Mudaffara is similar to Gibna bayda except even more salt is added to the milk. Rennet or a rennet extraction is also added to achieve firm coagulation into curds within 4–6 h. Ripening takes place while the curds are submerged in the whey. The curds are then transferred to wooden moulds lined with cheese cloth or muslin and the whey is allowed to drain out overnight. The drained whey is collected into a clean pan and boiled for 15 mins prior to removal of any fats and coagulated whey proteins. Then starter from previously fermented milk is added and left overnight to ferment. The next day the cheese is removed from the moulds and cut into 10 cm cubes that are then immersed in the whey. Giban Mudaffara remains preserved for a long time because it is immersed in the whey (Salih et al. 2011).

Gouda is a yellowish cheese named after the city of Gouda in the Netherlands, and is one of the primary Dutch-type cheese varieties produced worldwide (Walstra et al. 1993). It is a semi-hard cheese manufactured from cow's milk, although sheep and goat's milk can also be used (Van den Berg 2000). This type of cheese ripens for 1–20 months during which time the flavour changes from mild to

strong and it can be consumed at any stage of maturity depending on the product characteristics required, the flavour preferences of consumers and economic factors (Walstra et al. 1993). There were ten species of LAB were isolated and identified from this product (Table 2.16).

Kefir is an alcohol containing milk from Caucasian countries such as Russia, Armenia, etc., fermented by yeasts and LAB. The colour of these products is white or yellowish and the texture is rather thick, but not gluey, with an elastic consistency (Tamime 2006). According to Polish Standards, the microscopic composition should be 80 % *Lactobacillus* spp., 12 % yeasts and 8 % *Lactococcus* spp.. The LAB composition reported includes over 30 species or subsp. species of LAB and 24 species of yeasts (Tamime 2006). *Pseudomonas* spp. and *E. coli* have also been detected in kefir by DGGE (Chen et al. 2008).

Koumiss is produced from mare's milk that has been naturally fermented by LAB and yeasts and is made in the south of Russia, Mongolia and the northwestern regions of China. There have been 62 species or subspecies of LAB recorded from koumiss (Table 2.10) and in other sections of Chap. 7. Isolates of LAB reported include four species of Enterococcus, 45 species or subspecies of *Lactobacillus*, four species of *Lactococcus*, four species of *Leuconostoc*, two species of *Streptococcus* and three species of *Weissella* (Burentegusi 2002; Uchida et al. 2007; Watanabe et al. 2008).

Kule naoto is traditionally fermented milk produced by the Maasai tribespeople of Kenya. It is spontaneously fermented from raw cow's milk in traditionally prepared calabashes. The calabashes are first treated with burnt smoky twigs from a traditional plant. The milk is further pre-treated by adding fresh cow's blood, before initiating the fermentation process (Mathara et al. 2004, 2008). Seven species of LAB have been isolated from the fermented products (Table 2.4). Further studies have shown that the LAB composition of Kule naoto was 55 % from the genus *Lactobacillus*, 14 % from *Lactococcus*, 25 % from *Enterococcus* and 6 % from *Leuconostoc*. *Lactobacillus plantarum* was the predominant species and constituted 60 % of the Lactobacillus species present (Mathara et al. 2004).

Kurut is a traditional naturally fermented yak's milk product is one of the staple foods of the nomadic people from the Tibet Autonomous region of China. It is also consumed by people from Qinghai, Gansu and Sichuan Provinces of China. There have been 19 species of LAB isolated from kurut (Table 2.16) and the predominant species were *S. thermophilus*, *Lb. helveticus*, *Lb. fermentarum* and *S. thermophilus* in the Sichuan Province, Gansu Province, Tibet Autonomous Region and Qinghai Province of China, respectively (Sun et al. 2010; Bao et al. 2010, 2011; Liu et al. 2009).

Mish is a fermented cow's milk product from both the state of Darfur in western Sudan and from Egypt. Although most associated with Sudan mish is thought to have been introduced from Egypt (Abdelgadira et al. 1998). The preparation method of mish from Darfur is more similar to that of the product from Egypt than another Sudanese dairy product, biruni. Mish is produced by boiling the milk then cooling it and inoculating with a small quantity of material from a

previous batch as a starter. After souring, seeds of black cumin (*Nigella sativa*) and fenugreek (*Trigonella foenum graecum*) are added. Sometimes, a few pods of green or red pepper are also added. The product is fermented for a further two or more days before consumption (Dirar 1993).

Milk cake is a traditional fermented cheese-like product made from goat's milk and consumed by ethnic minority communities in the Yunnan province of China. The nutritional value of goat's milk is very high and includes vitamins and a diversity of trace elements (Chen et al. 2009). LAB (from 8.32×10^{10} CFU/g to 10.16×10^{10} CUF/g) and yeast (from 1.06×10^{10} CUF/g to 6.15×10^{10} CUF/g) were the predominant microorganisms in milk cake (Bao et al. 2011). Totally, 76 strains of LAB were isolated from 13 samples of milk cake and attributed to 9 species based on conventional and 16S rRNA gene sequence analysis methods (Bao et al. 2011). *Lactococcus lactis* subsp. *lactis* and *Lc. garvieae* were the most abundant species of LAB in Yunnan milk cake.

Nunu is yogurt-like product prepared and consumed by the Fulani people in Ghana. Nunu is processed from fresh cow's milk in calabashes or rubber buckets by spontaneous fermentation. The production method of this products was described in literature by Akabanda et al. (2010) The predominant LAB present included different species of *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Streptococcus*.

Philu is an indigenous cream-like milk product produced from cow's or yaks milk, and is typically cooked and eaten as a delicacy with boiled rice in Bhutan and the Indian state of Sikkim. During philu preparation, fresh milk is collected in cylindrical bamboo or wooden vessels and slowly swirled around the walls of the vessels for 5–10 min. During this time, a creamy mass begins to stick to the walls of the vessels. The remaining liquid is poured off and the vessels upturned to drain out the remaining liquid. This process is repeated daily for 6–7 days until a thick, white creamy layer is formed on the inside walls of the vessels. This soft mass is the philu and it is scraped off and stored in a dry place for consumption. Philu produced from cow's milk is white in colour with a butter-like texture and slightly bland taste, while philu produced from yak's milk has a more creamy-white colour and an inconsistent semi-solid texture. Philu is a high-priced traditional milk product sold in local markets of Sikkim and Bhutan. Chhu or sheden is a strong-flavoured indigenous cheese-like product prepared in a similar way from cow's milk or yak's milk in Sikkim, the Darjeeling hills, Arunachal Pradesh and Ladakh in India, Nepal, Bhutan and the Tibet Autonomous region of China (Dewan and Tamang 2007).

Qula is a grainy, hard, yellow or white cheese made from yak's milk in the Tibetan plateau of China. The process of making Qula cheese by traditional Tibetan methods has been described by Duan et al. (2008). LAB were the most important group of microorganisms in Qula (Table 2.16) and, of the 15 species or subspecies of LAB identified, *Lb. helveticus* was the predominant species.

Rob, also known as roub or robe, is produced in rural households of Sudan by fermentation of cow's milk or sheep and goat's milk. Surplus milk is collected in a container, inoculated with a starter culture from the previous day and left to

ferment overnight. The fermentation process usually starts in the evening when the animals return from grazing and the resulting sour product is churned in the morning when the herd leaves for grazing. Freshly produced rob has a pleasant taste with a pH of about 4.5. The early literature by Saeed (1981) and El Mardi (1988) revealed that *S. thermophilus*, *Lb. bulgaricus*, *Lb. helveticus*, *Lb. fermentum* and *Lc. lactis* were the most common LAB in rob. Hamza et al. (2009) also identified *Lb. delbreuckii* subsp. *bulgaricus*, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. casei* and *Lb. pentosus* using API 50CHL and *A. viridians*, *E. faecium*, *E. gallinarum*, *Lc lactis* sub sp. *lactis*, *Leuconostoc* sp., *S. acidominimus* and *S. bovis* by API 20 STREP. The results were confirmed by Random Amplified Polymorphic DNA (RAPD).

2.4.4 LAB in Fermented Vegetables and Beverages

2.4.4.1 LAB in Sourdough

Sourdough is an important modern fermentation method for cereal flours and water based upon an earlier spontaneous process (Vogel et al. 1999). The sourdough starter is used in the manufacture of a variety of products such as breads, cakes, Chinese steamed buns (mantou) and Chinese steamed stuffed buns (bao zi). Many sourdough wheat breads and cakes originate in Mediterranean countries, the San Francisco bay area of the United States and Southern America, whereas numerous sourdough products made with rye, wheat, barley or mixed flours originate in Germany, Central and Eastern Europe and Scandinavia (Stephan and Neumann 1999a, b). In Italy, wheat sourdough is used in more than 30 % of bakery products (Ottogalli et al. 1996). Baked (cake) and steamed products using wheat sourdough are staple foods in northern China. Due to their artisan and region-dependent preparation methods, sourdoughs have a very high diversity of LAB and yeast species and strains (Table 2.17). Recent studies suggest that more than 50 species of LAB, particularly species from the genus *Lactobacillus*, and more than 20 species of yeast, particularly from the genera *Saccharomyces* and *Candida*, can be in sourdough cultures used for making traditional/typical leavened baked goods worldwide (Corsetti et al. 2001; Minervini et al. 2012; Palomba et al. 2011; Venturi et al. 2012; Lattanzi et al. 2013).

2.4.4.2 LAB in Pickled Vegetables

Fermented vegetables play an important role in Asian family diet as a popular dish or as seasoning for food. Fermented vegetables have different names associated with the different materials and processes used to make them and the different countries and regions in which they are made. These include 'kimchi' (Korea and Japan), 'suan cai' or 'suan-tsai' (China or Tanwan) and 'sauerkraut' or 'kraut' (Germany).

Table 2.17 Species of LAB and yeasts found in sourdough products from around the world

Country	Product	Species of LAB present	Methods for isolation and identification	Reference
Belgium	Wheat and rye sourdoughs	11, 36, 38, 45	Polyphasic	Scheirlinck et al. (2009)
		36, 38, 39, 45		Scheirlinck et al. (2007)
		17, 27, 35, 36, 38, 39, 43, 45		Scheirlinck et al. (2008)
Denmark	Rye sourdough	39, 41, 45	Phenotypic	Rosenquist and Hansen (2000)
Finland	Rye sourdough	9, 13, 27, 38	Phenotypic	Salovaara and Katunpää (1984)
France	Wheat bread	11, 13, 18, 20, 22, 38, 56b, 62	Phenotypic	Infantes and Tourneur (1991)
Germany	Wheat sourdough	10b, 12, 13, 20, 24, 38	Phenotypic	Spicher (1959)
Greek	Traditional wheat sourdoughs	11, 39, 45, 68	SDS-whole cell protein/DNA-PCR 16 rRNA sequencing	Vuyst et al. (2002)
	Rye bread	9, 11, 13, 24, 38, 45	Phenotypic	Spicher (1979, 1987)
	Rye sourdough	9, 10, 11, 12, 13, 24, 38, 45	Phenotypic	Spicher (1984)
	Wheat sourdoughs (Panettone, wheat bread)	11, 13, 38, 28, 71	Phenotypic	Spicher (1987)
	Rye sourdough	10b, 24b, 39, 41	RAPD-PCR	Müller et al. (2001)
	Rye bran	10b, 33, 16, 39, 24, 24b, 25b, 41	PCR-DGGE	Meroth et al. (2003)
	Wheat and rye sourdoughs	11, 12, 17, 18, 26, 27, 34, 35, 36, 38, 39, 43, 44, 45, 55, 58, 62, 68, 69	PCR-DGGE, AFLP	Scheirlinck et al. (2008)

(continued)

Table 2.17 (continued)

Country	Product	Species of LAB present	Methods for isolation and identification	Reference
Italy	Panettone, Brioche	11, 38	Phenotypic	Galli and Ottogalli (1973)
	Panettone, Brioche	24, 38, 45, 55	Phenotypic	Galli et al. (1988)
	Umbrian wheat sourdoughs	38, 45	Phenotypic	Gobbetti et al. (1994)
	Pizza (Naples)	38, 44, 55	Phenotypic	Coppola et al. (1996)
	Verona sourdoughs	45	RAPD-PCR	Zapparoli et al. (1996)
	Apulian wheat sourdoughs	9, 10, 11, 20, 24, 38, 45, 51, 53, 69	16S rDNA sequencing and 16S/23S rRNA spacer region PCR	Corsetti et al. (2001)
Iran	Sangak	11, 38, 55	Phenotypic	Azar et al. (1977)
Mexico	Pozole (maize)	10, 13, 19, 38, 50	16S rDNA sequencing	Escalante et al. (2001)
Morocco	Sourdough ferments and traditional starters	11, 12, 13, 38, 55	Traditional methods	Boraam et al. (1993)
	Sponges	13, 55 and *Pediococcus* sp.	Phenotypic	Faid et al. (1994)
	Soft wheat flour	12, 13, 19, 38, 45, 55, 62	Phenotypic	Faid et al. (1994)
Portugal	Broa	1, 2, 4, 11, 18, 19, 51	Phenotypic	Rocha and Malcata (1999)
Russia	Rye sourdough	11, 25, 38	Phenotypic	Kazanskaya et al. (1983)
Spain	Wheat sourdough	11, 38	Phenotypic	Barber et al. (1983)
	Wheat sourdough	11, 38	Phenotypic	Barber et al. (1983)
Sudan	Kisa (sorghum sourdough)	10b, 24, 41	Molecular and traditional method	Hamad et al. (1997)

(continued)

Table 2.17 (continued)

Country	Product	Species of LAB present	Methods for isolation and identification	Reference
	Kisa	3, 24, 27, 50	RAPD	
Sweden	Rye/Wheat	9, 11, 19, 24, 38, 42, 45, 71	Phenotypic	Spicher (1987)
	Rye sourdough	*Lactobacillus* sp., 62	Phenotypic	Lönner et al. (1986)
USA	San Francisco sourdough French bread	45	Phenotypic	Kline and Sugihara (1971)
China	Traditional sourdough	2, 11, 18, 17, 24, 27, 33, 36, 38, 43, 45, 50, 53, 55, 68, 69	16S rRNA genes sequences and DGGE analysis	Zhang et al. (2011)

Note 1. *E. casseliflavus*, 2. *E. durans*, 3. *E. faecalis*, 4. *E. faecium*, 5. *E. gallinarum*, 6. *E. italicus*, 7. *E. thailandicus*, 8. *Lb. acetotolerans*, 9. *Lb. acidophilus*, 10. *Lb. alimentarius*, 10b. *Lb. amylovoru*s, 11. *Lb. brevis*, 12. *Lb. buchneri*, 13. *Lb. casei*, 14. *Lb. confuses*, 15. *Lb. coryniformis* subsp. *torquens*, 16. *Lb. crispatus*, 17. *Lb. crustorum*, 18. *Lb. curvatus*, 19. *Lb. delbrueckii* subsp. *bulgaricus*, 20. *Lb. delbrueckii* subsp. *delbrueckii*, 21. *Lb. delbrueckii* subsp. *indicus*, 22. *Lb. delbrueckii* subsp. *lactis*, 23. *Lb. diolivorans*, 24. Lb.f*Fermentum*, 24. *Lb. frumenti*, 25. *Lb. graminis*, 25b. *Lb. johnsonii*, 26. *Lb. hamsteri*, 27. *Lb. helveticus*, 28. *Lb. hilgardii*, 29. *Lb. kefiranofaciens* subsp. *kefiranofaciens*, 30. *Lb. kefiranofaciens* subsp. *kefirgranum*, 31. *Lb. kefirgranum*, 32. *Lb. kefiri*, 33. *Lb. mindensis*, 34. *Lb. parabuchneri*, 35. *Lb. paracasei*, 36. Lb. *paralimentarius*, 37. *Lb. pentosus*, 38. *Lb. plantarum*, 39. *Lb. pontis*, 40. *Lb. rapi*, 41. *Lb. reuteri*, 42. *Lb. rhamnosus*, 43. *Lb. rossia*, 44. *Lb. sakei*, 44a. *Lb. salivarius*, 45. *Lb. sanfrancisco*, 46. *Lb. spicheri*, 47. *Lb. sunkii*, 48. *Lb. uvarum*, 49. *Lc. garvieae*, 50. *Lc. lactis* subsp. *cremoris*, 51. *Lc. lactis* subsp. *lactis*, 52. *Lc. raffinolactis*, 53. *Leuc. citreum*, 54. *Leuc. lactis*, 55. *Leuc. mesenteroides*, 56. *Leuc. mesenteroides* subsp. *mesenteroides*, 56b. *Leuc. mesenteroides* subsp. *dextranicum*, 57. *Leuc. pseudomesenteroides*, 58. *P. acidilactici*, 59. *P. damnosus*, 60. *P. ethanolidurans*, 61. *P. parvulus*, 62. *P. pentosaceus*, 63. *S. bovis*, 64. *S. parauberis*, 65. *S. thermophilus*, 66. *S. mutans*, 67. *S. salivarius*, 68. *W. cibaria*, 69. *W. confuse*, 70. *V. carniphilus*, 71. *W. viridescens*, *W. paramesenteroides*

A. *Corynebacteria pseudodiptherium*, B. *Saccharomyces cerevisiae*, C. *Candida kefyr*, D. *Cadida lusitaniae*, E. *Candida colliculosa,* F. *Kluyveromyces maxianus,* G. *Debaryomyces hansenii,* H. *Pichia farinose,* I. *Pichia guilliermondii,* J. *Pichia anomala,* K. *Candida humilis,* L. *Rhodotorula mucilaginosa*

Kimchi is a traditional fermented vegetable from Korea. Most kimchi is characterised by its hot taste because of the fairly high quantities of chilli pepper used. Lactic acid bacteria (LAB), including *Lb. plantarum*, *Lb. brevis*, *Lb. acidophilus*, *Lb. homohiochii*, *Leuconostoc* species and *Lc. lactis* have all been isolated from Korean kimchi (Lee et al. 1999; Park et al. 2010). The most abundant LAB is the hetero-fermentative species *Leuc. mesenteroides*, particularly at the initial to the middle stages of fermentation. However, the total number of this species decreases sharply as fermentation proceeds and the pH drops below 4.0. At the final fermentation stages the homofermentative species *Lb. plantarum* predominates (Mheen and Kwon 1984). In the last 10 years, ten further species have been recorded from kimchi: *Lb. kimchii* (Yoon et al. 2000), *Lb. xiangfangensis* (Gu et al. 2013), *Lb. futsaii* (Chao et al. 2012), *Lb. plantarum* subsp. *plantarum* (Bringel et al. 2005), *Lb. kimchicus* (Liang et al. 2011), *Lb. koreensi* (Bui et al. 2011), *Lc. kimchii* (Kim et al. 2002), *Lc. inhae* (Kim et al. 2003), *W. kimchii* (Choi et al. 2002) and *W. koreensis* (Lee et al. 2002).

Suan cai (pickled vegetable), also known as fu tsai, pao cai or suan-tsai, is a very popular food in western and northeastern China and Taiwan. There are more than 11 different types of fermented vegetables or pickled vegetables in China (Hui et al. 2012). In recent years, the diversity of LAB has been determined using the 16S rRNA genes sequence and housekeeping gene sequence (dnaA, pheS and rpoA) analysis (Chen et al. 2006a; Chao et al. 2009). Eighteen species of LAB from five genera have been reported from suan cai from Taiwan, including *Enterococcus* (1 species), *Lactobacillus* (11 species), *Leuconostoc* (3 species), *Pediococcus* (1 species) (Chao et al. 2009).

2.4.4.3 LAB in Traditional Sausage and Fermented Meat-Based Food

Meat plays an important role in people's diet but it requires special storage measures since it is highly sensitive to microbial spoilage. Traditional methods for preservation of meat are drying, salting and fermentation. Worldwide, there is a vast variety of fermented food based on meat and fish. For example, 330 different types of sausages are produced in Germany alone (Lerche 1975). Fermented sausages are common products throughout world, and they are made using a diversity of production methods resulting in characteristics unique to the different countries or regions of the same country from which they originate (Table 2.18). It is well known that in the traditional fermentation process bacteria, yeast and fungi work in combination and affect the final quality of the fermented sausage. LAB are regarded as having an important contribution to the ripening process (Hammes et al. 1990) and they vary in species composition between different products (Table 2.17). Their essential role in this process was recognised first in the U.S.A. where patents for the application of *Lactobacillus plantarum*, *Lb. brevis* and *Lb. fermenti*, were obtained by Jensen and Paddock (1940). A thorough investigation of LAB in both commercial products and in ripening sausages showed that the dominant LAB were psychrophilic atypical streptobacteria (Reuter 1967).

Hammes (1985) analysed 37 samples from 12 suppliers to the German market and identified LAB including *P. acidilactici, P. penteosaceus, Lb. plantarum, Lb. sake* and *Lb. survatus* (Hammes 1985). Cocolin et al. (2004) studied the ecology of fresh sausages and characterised populations of LAB using a polyphasic approach after different storage times at 4 °C. The results showed that *Brochothrix thermosphacta* and *Lb sakei* were the most abundant species present. In particular, *B. thermosphacta* was present throughout the process, as determined by both DNA and RNA analysis. Other bacterial species, mainly *Staphylococcus xylosus, Leuc. mesenteroides* and *Lb. curvatus*, were detected by DGGE. Moreover, after different storage times, different LAB species were identified, including *Lc. lactis* subsp. *lactis*, *Lb. casei*, *E. casseliflavus*, *Leuc. mesenteroides* and *Lb. sakei*.

2.4.5 LAB in Other Fermented Food

There are numerous plant-based fermented food products that are not pickled leaf vegetables as described above. These products are fermented from beans and other seeds, such as rice, maize and millet or root vegetables. LAB was involved in all of these processes, influencing the characteristics of flavour and texture, and also the probiotic qualities of the final product (Table 2.18). There are many kinds of beverage produced from fermented cereals. Dolo and pito are two similar West African traditional fermented beverages produced from sorghum grains that are popular drinks. They have contributed to the diet of people in West Africa together with other fermented foods for centuries (Odunfa 1985). Pito is common in Ghana, Togo and Nigeria, whereas dolo and other similar products (e.g. tchapalo and tchoukoutou), are common in Burkina Faso, Ivory Cost, Mali and Benin (Yao et al. 1995; Konlani et al. 1996; Kayode et al. 2004). Tarhana and tarhana-like products are traditional Turkish fermented cereal-based food products, which are well known under different names in the other countries, e.g. kishk in Syria, Palestine, Jordan, Lebanon and Egypt; talkuna in Finland; kushuk in Iraq and Iran; thanu in Hungary and trahanas in Greece (Siyamoğlu 1961; İbanoğlu and İbanoğlu 1999; Blandino et al. 2003). While their preparation techniques vary depending on region they are similar; after mixing all the ingredients dough is formed that is allowed fermenting for 1–7 days at room temperatures (25–30 °C). The fermentation is followed by drying and grinding. Yeast and LAB are the predominant microorganisms but the species composition and abundance varies between products (Table 2.19).

2.4.6 Prospects and Challenges

Throughout history traditional fermented foods have been regarded as the main source of nutrition for many rural communities and nomadic people. They

Table 2.18 Species of LAB found in fermented meat and fish-based products from around the world

Country/region	Type of fermented product	Name of product	Method for identification	Species of LAB present	References
Turkey		Sucuk	Traditional culture-dependent and culture indepedent (rep-PCR fingerprinting and DGGE	*Lb. alimentarius, Lb. plantarum, Lb. brevis, Lb. farciminis, Lb. saket, Lb. curvatus, Lc. piscium, W. halotolerans, Leuc. mesenteroides, Leuc. citreum, W. viridescens*	Adewumi et al. (2013)
Spain	Sausages	Chorizo, fuet, salchichon	RAPD-PCR	***Lb. sakei*** *Lb. curvatus* *Leuc. mesenteroides*	Aymerich et al. (2006)
Italy	Fresh sausages	Sausages	Traditional culture-dependent method and DGGE	*Lb. sakei* *Lb. curvatus* *Lb. casei* *Lc. lactis* subsp. *lactis* *Leuc. mesenteroides*	Cocolin et al. (2004)
Italy	Fermented sausages	Sausages	RAPD-PCR and DGGE	***Lb. sakei, Lb. curvatus,*** *Lb. brevis*, *Lb. casei*, *Lb. plantarum*, *Lb. paraplantarum*, *Lc. garvieae*, *Lc lactis*, *Leuc. carnosum*, *Leuc. mesenteroides*. *W. hellenica*, *W. paramesenteroides*	Urso et al. (2006)
Côte d'Ivoire	Fermented fish	Adjuevan	Traditional culture-dependent method and DGGE	*Lb. fermentum*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus*, *Lc. garviae*, *Lc. raffinolactis*, *Leuc. lactis*, *P. pentosaceus*	Clementine et al. (2012)
Italy	Fish product	Seafood salad	RAPD-PCR	*Lb. curvatus*, *Lb. malefermentans*, *Lb. plantarum*, *Lb. sanfranciscensis*, *C. piscicola*, *E. faecalis*, *Lc. lactis*, *Leuc. mesenteroides*, *Weissella* sp.	Andrighetto et al. (2009)

currently play an important role in improving the economy, finance and business of local societies. However, with accelerating urbanisation and a reduction in the nomadic life style, there are fewer and fewer traditional fermented foods routinely prepared traditionally at home. In addition, in order to meet the demand for traditional products by urban populations, and to improve food quality, safety and efficiency of production, these household fermentation technologies have been advanced to an industrial scale. However, this transition from small-scale household production to industrial manufacturing has been a challenge for food microbiologists. Advances have been made in our understanding of the species richness, diversity and behaviour of the microbial species present in fermented food. However, more work should be done to identify more LAB, to increase our knowledge of the mechanisms of fermentation, to understand the roles of each LAB species and to characterise probiotic bacteria.

2.5 Review of Biodiversity of Lactic Acid Bacteria in Silage

Silage is currently the most commonly preserved cattle feed in many countries (Cai 1999). Lactic acid bacteria (LAB), including rod and cocci (Fig. 2.9), play an important role in silage fermentation. LAB, which are microorganisms that naturally exist on forage crops, are responsible for silage fermentation and the occurrence of dry matter (DM) loss and proteolysis during storage. LAB commonly grow with other plant-associated microorganisms during silage fermentation, and they generally define the fermentation characteristics of silage. Moist dairy farm silage is based on natural lactic acid fermentation. The epiphytic LAB transform the water-soluble carbohydrates into organic acids during the ensiling process. As a result, the pH is reduced, and the forage is preserved. The fermentation quality may be influenced by the diversity, quantity and activity of the epiphytic LAB (Lin et al. 1992). Some research workers realised that most of the epiphytic LAB was caused by heterofermentation, which may not have positive organisms for lactic acid-dominating fermentation in the silo (Cai 1999).

As shown in Table 2.1, the natural fermentation processes in silages of corn, sorghum, forage paddy rice and alfalfa are dominated by species of *Weissella (W), Leuconostoc (Le.), Lactococcus (La.)* and *Lactobacillus (L.)*. Some isolates from forage crops and grasses have been identified as species of lactobacilli, enterococci, pediococci, weissella, lactococci and leuconostocs. Cai (1999), Lin et al. (1992) and Pang et al. (2011) examined a large number of LAB isolated from forage crops and grasses (Fig. 2.10, Table 2.20) and found that the predominant LAB were lactic acid-producing cocci and that the least numerous LAB were lactobacilli (mostly homofermentative). Ennahar et al. (2003) also found that although all LAB groups were present in paddy rice silage, homofermentative lactobacilli and lactococci and heterofermentative leuconostocs were present in greater numbers.

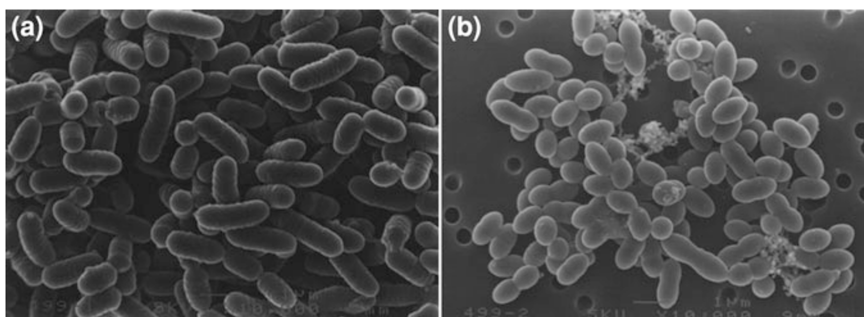


Fig. 2.9 Electronic-microscope of *Lactobacillus plantarum* (**a**, rod) and *Lactococcus lactis* (**b**, cocci) isolated from silage.

The lactobacilli play a more important role in the fermentation processes and effectively promote lactic acid fermentation for a longer period of time than lactic acid-producing cocci, e.g. enterococci, streptococci, leuconostocs, weissella and pediococci. Generally, when the lactobacilli reach a level of at least 10^5 colony forming units (cfu)/g fresh matter (FM), silage can be well preserved. *L. casei* and *L. plantarum* are usually found living in association with forage crops and silage. Many studies have been conducted on lactobacilli as the dominant microbial population on forage crops and farm silage (Cai 1999). The predominance of *L. plantarum* has been reported elsewhere (identification based on only 16S rDNA sequences and carbohydrate utilisation) (Cai 1999). Further, precise differentiation among these species was not conducted in silage samples. In addition, little is known about the prevalent subspecies of *L. plantarum*, which play a major role in silage fermentation. The most frequently presented microorganism, *L. coryniformis,* has often been found on plant material and in various silages. The presence of *L. curvatus* in sorghum silages has previously been reported, and its inoculation is highly effective in preserving silages of Italian ryegrass. The identification of *L. acidipiscis* and *L. sakei* subsp. *carnosus* from forage crops was reported as useful for understanding their range of ecological niches.

The cocci, such as leuconostocs, pediococci and weissella strains, have been isolated at low frequencies in forage crops and their silages (Cai 1999; Lin et al. 1992). Certain *Weissella* (*W.*) isolates from silages have been identified as *W. paramesenteroides* (Cai 1999). The isolation of *W. hellenica* by Tohno et al. (2012) provides the first evidence that the natural habitats of this species are not only meat and meat products (Collins et al. 1993) but also vegetative forage crops with mixed pastures of timothy and orchardgrass. *W. cibaria*, was also found as epiphytic on corn stover by Pang et al. (2011, 2012). LAB strains, such as *Leuconostoc* and *Pediococcus*, have been isolated at low frequencies in forage crops and their silages (Cai 1999; Lin et al. 1992). They are also widely used as starter cultures or control barriers for food pathogens in vegetables. Pediococci are often found living in association with plant material, dairy products and foods produced by LAB (Cai

Table 2.19 LAB in other plant-based traditional fermented food from around the world

Country/Region	Name of fermented product	Type of products	Raw material	Species of LAB	Counts of LAB as CFU/g	Reference
Zimbabwe	Chimera	Non-alcoholic and alcoholic beverages	Sorghum, bullrush millet or finger millet		ND	
Taiwan	Dochi	Seasoning for food	Black bean	4, 58, 72	4×10^1 to 1.1×10^7	Chen et al. (2006a)
Turkey	Tarhana	Cereal-based food	Wheat flour and yogurt	4, 13, 19, 24, 38, 57, 58, 65	1×10^3 to 1×10^6 varied depending on length of fermentation	Chen et al. (2006b) Sengun et al. (2009)
Mexico	Pozole	Maize dough	Maize	13, 20, 24, 38, 63	1×10^7 to 1×10^9 varied depending on length of fermentation	Omar and Ampe (2000)
Japan	Nukadoko	Fermented rice bran	Rice	8, 24, 10, 11, 19, 50, 65.		Sakamoto et al. (2011) Randazzo et al. (2002)
Africa	Gari	Fermented cassava	Cassava	11, 38, 57	1×10^6 to 1.8×10^9	Kostinek et al. (2005)
China	Sour congee	Fermented cereal gruel	Rice millet	3, 6, 11, 24, 35, 38, 51	ND	Yu et al. (2011)
China	Stinky tofu	Fermented chinese snack	Soybean	11, 12, 16, 17, 20, 24, 44, 50, 51, 53, 54, 58, 63, 65, 68, 69	1×10^5 to 1×10^7	Chaoi et al. (2008)
Ghana	Fermented cocoa beans	Raw material for chocolate	Cocoa beans	1, 4, 11, 24, 38, 50, 55, 57, 69		Camu et al. (2007)

(continued)

Table 2.19 (continued)

Country/Region	Name of fermented product	Type of products	Raw material	Species of LAB	Counts of LAB as CFU/g	Reference
China	Vinegar	Seasoning for food	Sorghum pea, millet, wheat bran			
Africa	Dolo and Pito	Fermented beverages	Sorghum	19, 20, 24, 51, 54, 58		Sawadogo-Lingani et al. (2007)

Note 1. *E. casseliflavus*, 2. *E. durans*, 3. *E. faecalis*, 4. *E. faecium*, 5. *E. gallinarum*, 6. *E. italicus*, 7. *E. thailandicus*, 8. *Lb. acetotolerans*, 9. *Lb. acidophilus*, 10. *Lb. alimentarius*, 11. *Lb. brevis*, 12. *Lb. buchneri*, 13. *Lb. casei*, 14. *Lb. confuses*, 15. *Lb. coryniformis* subsp. *torquens*, 16. *Lb. crispatus*, 17. *Lb. crustorum*, 18. *Lb. curvatus*, 19. *Lb. delbrueckii* subsp. *bulgaricus*, 20. *Lb. delbrueckii* subsp. *delbrueckii*, 21, *Lb. delbrueckii* subsp. *indicus*, 22. *Lb. delbrueckii* subsp. *lactis*, 23. *Lb. diolivorans*, 24. *Lb. fermentum*, 25. *Lb. graminis*, 26. *Lb. hamsteri*, 27. *Lb. helveticus*, 28. *Lb. hilgardii*, 29. *Lb. kefiranofaciens* subsp. *kefiranofaciens*, 30. *Lb. kefiranofaciens* subsp. *kefirgranum*, 31. *Lb. kefirgranum*, 32. *Lb. kefiri*, 33. *Lb. mindensis*, 34. *Lb. parabuchneri*, 35. *Lb. paracasei*, 36. *Lb. paralimentarius*, 37. *Lb. pentosus*, 38. *Lb. plantarum*, 39. *Lb. pontis*, 40. *Lb. rapi*, 41. *Lb. reuteri*, 42. *Lb. rhamnosus*, 43. *Lb. rossia*, 44. *Lb. sakei*, 45. *Lb. sanfrancisco*, 46. *Lb. spicheri*, 47. *Lb. sunkii*, 48. *Lb. uvarum*, 49. *Lc. garvieae*, 50. *Lc. lactis* subsp. *cremoris*, 51. *Lc. lactis* subsp. *lactis*, 52. *Lc. raffinolactis*, 53. *Leuc. citreum*, 54. *Leuc. lactis*, 55. *Leuc. mesenteroides*, 56. *Leuc. mesenteroides* subsp. *mesenteroides*, 57. *Leuc pseudomesenteroides*, 58. *P. acidilactici*, 59. *P. damnosus*, 60. P. *ethanolidurans*, 61. *P. parvulus*, 62. *P. pentosaceus*, 63. *S. bovis*, 64. *S. parauberis*, 65. *S. thermophilus*, 66. *S. mutans*, 67. *S. salivarius*, 68. *W. cibaria*, 69. *W. confuse*, 70. *V. carniphilus*, 71. *W. viridescens*, *W. paramesenteroides*

A. *Corynebacteria pseudodiptherium*, B. *Saccharomyces cerevisiae*, C. *Candida kefyr*, D. *Cadida lusitaniae*, E. *Candida colliculosa,* F. *Kluyveromyces maxianus,* G. *Debaryomyces hansenii*

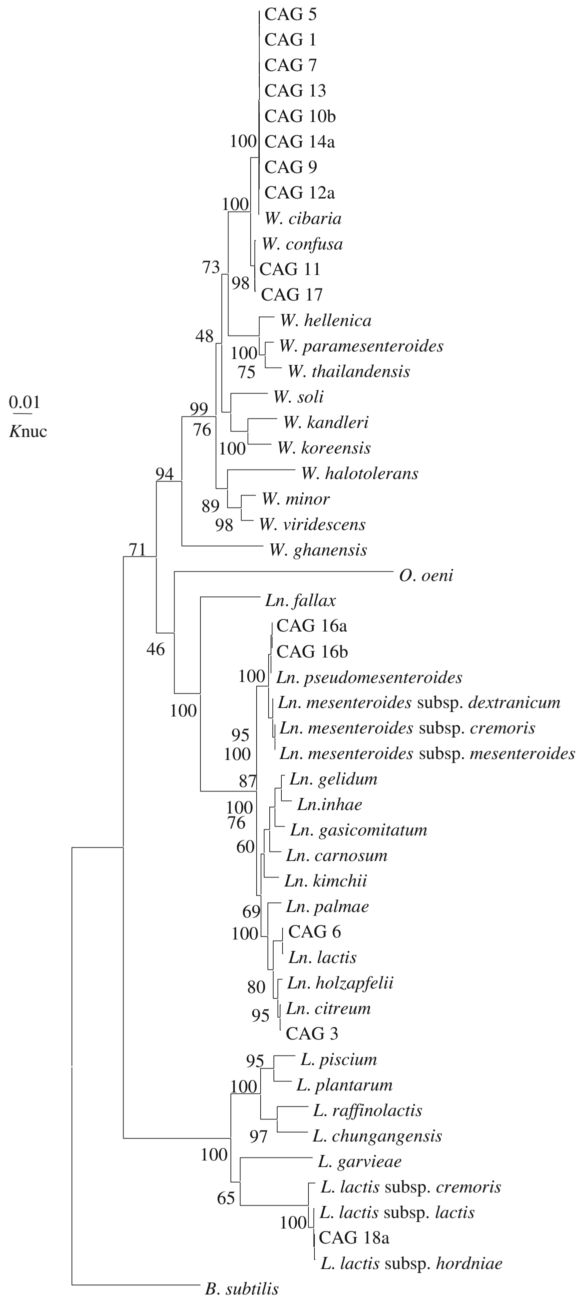


Fig. 2.10 Phylogenetic tree showing the relative positions of silage strains with *Weissella*, *Leuconostoc* and *Lactococcus* species, as inferred by the neighbour-joining method with 16S rRNA gene sequences. *B. subtilis* is used as an outgroup. Bootstrap values for a total of 1,000 replicates are shown at the nodes of the tree. The bar indicates 1 % sequence divergence. *K*nuc, nucleotide substitution rates. *W.*, *Weissella*; *Ln.*, *Leuconostocs*; *L.*, *Lactococcus*

Table 2.20 The distribution of Lactic acid bacteria on silages (CFU/g of FM)

Silage	*W. cibaria*	*W. confusa*	*Le. citreum*	*Le. lactis*	*Le. pseudomesenteroides*	*Lc. Lactis* subsp. *lactis*	*Lb. paraplantarum*	*Lb. plantarum*
Corn (Cultivar: Zhongyuandan 32)	4.6×10^7	ND	ND	ND	ND	ND	ND	ND
Corn (Nongda 108)	3.0×10^{s}	ND	ND	ND	ND	ND	ND	ND
Corn (Nongda 95)	6.0×10^7	ND	ND	ND	ND	ND	ND	ND
Corn (Yousi)	4.5×10^7	4.5×10^7	ND	ND	ND	ND	ND	ND
Corn (Tiandan)	2.6×10^{s}	ND	ND	ND	ND	ND	ND	ND
Sorghum (Tiangaoliang M-81)	ND	ND	0.2×10^9	ND	ND	ND	ND	0.4×10^9
Forage paddy rice (Shuidao 305)	0.3×10^8	0.3×10^8	ND	0.3×10^8	ND	0.3×10^8	ND	0.9×10^8
Alfalfa (Derfy)	ND	ND	ND	ND	0.5×10^7	ND	0.3×10^7	0.2×10^7

ND not detected; *CFU* colony forming unit; *FM* fresh matter
W., Weissella; Le., Leuconostocs; Lc., Lactococcus; Lb., Lactobacillus

Fig. 2.11 A dendrogram illustrating the relatedness of the ribotyping patterns of NJ 317^T among closely related species of the genus *Lactococcus*. The dendrogram was analysed by Pearson similarity coefficient analysis and UPGMA algorithm

1999; Lin et al. 1992), and several papers have reported pediococci as the dominant microbial population on forage crops and silage. Some isolates from forage crops and silage have been identified as *P. acidilactici* and *P. pentosaceus* (Lin et al. 1992). The presence of *Enterococcus* (*E.*) *faecalis* suggests a faecal origin for some PRS microorganisms. The natural habitat of lactococci is milk, but *La. lactis* subsp. *lactis* has been isolated previously from plants, vegetables and cereals. Native LAB populations on plants are not the same from crop to crop. Native LAB levels are generally lower in alfalfa (*Medicago sativa* L.), perennial grasses (10^5 cfu/g FM and greater) or (10^6 cfu/g FM), corn (*Zea mays* L., 10^7 cfu/g FM) and sorghum (*Sorghum bicolor* L. Moench, 10^7 cfu/g FM) (Pahlow et al. 2003). Environmental conditions also have an effect on native LAB levels. In alfalfa, native LAB was higher in warmer temperatures, after a longer wilting time and when rainfall occurred during wilting (Muck 1989). Moreover, native LAB is low in the standing crop and increases exponentially after chopping in both corn and alfalfa (Lin et al. 1992).

The populations of epiphytic LAB in farm silage are not always large enough or do not have a composition suitable for promoting efficient homo-lactic fermentation. The effectiveness of silage inoculants depends upon the quality (growth rate and environmental adaptability) and quantity of the microorganisms used, among other things. In general, favourable improvements in the rate of pH decline and increased lactic acid levels have been noted with legumes, grasses and cereal silages. In previous investigations of Cai et al. (1999), the inoculation of various forages with homofermentative *Lactobacillus* spp. (predominantly *L. plantarum*) in combination with *Pediococcus*, *Enterococcus* or *Lactococcus* spp. had beneficial effects (Fig. 2.11).

The genetic interrelationships of the silage strains and related LAB have been studied extensively by using Ribotyping, 16S rDNA sequence analysis and DNA–DNA hybridisation experiments, and some new species, such as *Lactococcus fujiensis* from Chinese cabbage silage and *Lactobacillus nasuensis* from sudangrass silage, have been added by Cai et al. (2011, 2012). As shown in Fig. 2.3, the ribotyping patterns revealed that strains NJ 317, NJ 414 and NJ 415 were well separated from the reference *Lactococcus* species.

Table 2.21 Characteristics and diversity of lactic acid bacteria strains isolated from silage

Character	Group A CAG 1	Group B CAG 11	Group C CAG 3	Group D CAG 6	Group E CAG 16a	Group F CAG 18a	Group G CAG 20	Group H CAG 18b
Shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Rod	Rod
Gram stain	+	+	+	+	+	+	+	+
Catalase	–	–	–	–	–	–	–	–
Gas from glucose	+	+	+	+	+	–	–	–
Optical form of lactate	D(−)	D(−)	D(−)	D(−)	D(−)	L(+)	DL	DL
Fermentation type	Hetero	Hetero	Hetero	Hetero	Hetero	Homo	Homo	Homo
Growth at temperature (°C):								
10	–	–	+	+	+	–	+	+
15	+	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+	+
45	–	–	–	–	–	–	–	–
Growth in NaCl:								
3.0 %	+	+	+	+	+	+	+	+
6.5 %	–	–	–	–	–	–	w	–
Growth at pH:								
3.0	–	–	–	–	–	–	w	–
3.5	–	–	–	–	–	–	+	w
4.0	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+

+, 90 % or more of the strains positive; −, 90 % or more of the strains negative; *w* weakly positive; *Homo* homofermentative; *Hetero* heterofermentative

[a] Group F strain was able to produce y-aminobutyric (GABA) from L-monosodium glutamate, and grow in 0.1 % bile salt

Table 2.22 DNA base compositon and DNA relatedness among *Lactococcus fujiensis* and phylognetically closely related *Lactococcus* species

Strain	G+C content (mol%)	DNA–DNA reassociation (%) with NJ 317^T	JCM 5805^T	JCM 10343^T
NJ 317^T	42.1	100.0	15.4	15.6
NJ 414	42.2	96.6	17.7	12.4
NJ 415	42.5	98.2	17.6	9.8
Lactococcus lactis subsp. *lactis* JCM 5805^T	39.4	20.2	100.0	13.2
Lactococcus lactis subsp. *hordniae* JCM 1180^T	nd	11.7	85.6	10.4
Lactococcus lactis subsp. *cremoris* JCM 16167^T	nd	9.4	80.2	17.0
Lactococcus garvieae JCM 10343^T	44.3	12.3	8.4	100.0
Lactococcus piscium JCM 11055^T	38.5	8.6	7.8	18.6
Lactococcus plantarum JCM 11056^T	nd	10.3	10.3	8.6
Lactococcus raffinolactis JCM 5706^T	41.5	11.3	12.4	10.0

JCM Japan Collection of Microorganisms. ^{T} type strain. *nd*, no data

These strains shared similar phenotypic characteristics and exhibited intragroup DNA homology values of over 96.6 %, which indicates that they are a single species. The G+C contents of the DNA for the strains were 42.1–42.5 mol%. DNA–DNA hybridisation indicated that these strains had low levels (<20.2 %) of DNA relatedness with *Lactococcus lactis*, *Lactococcus garvieae* and other type strains of previously described species, which shows that they were different species (Table 2. 21). Therefore, these strains, which are isolated from the silage, are placed in the genus *Lactococcus* as a new species, *Lactococcus fujiensis* (Table 2.22).

References

Abdel Moneim ES, Abdalla AI, Ahmed AF. Chemical and microbiological quality of Garris Sudanese fermented camel's milk product. Food Sci Technol. 2006;42:321–328.

Abdel Gadira WS, Ahmeda TK, Dirar HA. The traditional fermented milk products of the Sudan. Int J Food Microbiol. 1998;44:1–13.

Abriouel H, Martin-Platero A, Maqueda M, Valdivia E, Martinez-Bueno M. Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. Int J Food Microbiol. 2008;127:200–8.

Adams MR. Safety of industrial lactic acid bacteria. J Biotechnol. 1999;68:171–8.

Adewumi GA, Oguntoyinbo Folarin A, Keisam S, Romi W, Jeyaram K. Combination of culture-independent and culture-dependent molecular methods for the determination of bacterial community of iru, a fermented Park iabiglobosa seeds Frontiers in Microbiol. 2013;3(2):436.

Afzal MI, Jacquet T, Delaunay S, Borges F, Millière JB, Revol-Junelles AM, Cailliez-Grimal C. Carnobacterium maltaromaticum: identification, isolation tools, ecology and technological aspects in dairy products. Food Microbiol. 2010;27:573–9.

Aguirre M, Collins MD. Phylogenetic analysis of some *Aerococcus*-like organisms from urinary tract infections: description of *Aerococcus urinae* sp. nov. J Gen Microbiol. 1992;138:401–5.

Ahmed AM. The common microorganisms in the Sudanese white soft cheese. Sud J Vet Sci Anim Husb. 1997;36:109–12.

Ahmed IAM, Morishima I, Babiker EE, Mori N. Characterisation of partially purified milk clotting enzyme from Solanum dubium Fresen seeds. Food Chem. 2009;116:395–400.

Airidengcaicike, Chen X, Du XH, Wang WH, Zhang JC, Sun ZH, Liu WJ, Li L, Sun TS, Zhang HP. Isolation and identification of cultivable lactic acid bacteria in traditional fermented milk of Tibet in China. Int J Dairy Technol. 2010;63(3):437–44.

Akabanda F, Owusu-Kwarteng J, Glover LKR, Tano-Debrah K. Microbiological characteristics of Ghanaian traditional fermented milk product. Nunu. Nat Sci. 2010;8(9):178–87.

Alegria A, Alvarez-Martin P, Sacristan N, Fernandez E, Delgado S, Mayo B. Diversity and evolution of the microbial populations during manufacture and ripening of Casin, a traditional Spanish, starter-free cheese made from cow's milk. Int J Food Microbiol. 2009;136:44–51.

Alessandria V, Dolci P, Rantsiou K, Pattono D, Dalmasso A, Civera T, Cocolin L. Microbiota of the Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Cape Verde Islands. World J Microbiol Biotechnol. 2010;26:2211–21.

Andrewes FW, Ilorder TJ. A study of the Streptococci pathogenic for man. Lancct. 1906;ii:708–13.

Andrighetto C, Lombardi A, Ferrati M, Guidi A, Corrain C, Arcangeli G. Lactic acid bacteria biodiversity in Italian marinated seafood salad and their interactions on the growth of Listeria monocytogenes. Food Control. 2009;20:462–8.

Antunes A, Rainey FA, Nobre MF, Schumann P, Ferreira AM, Ramos A, Santos H, Da Costa MS. Leuconostoc ficulneum sp. nov., a novel lactic acid bacterium isolated from a ripe fig, and reclassification of *Lactobacillus fructosus* as *Leuconostoc fructosum* comb. nov. Int J Syst Evol Microbiol. 2002;52:647–55.

Aponte M, Fusco V, Andolfi R, Coppola S. Lactic acid bacteria occurring during manufacture and ripening of Provolone del Monaco cheese: detection by different analytical approaches. Int Dairy J. 2008;18:403–13.

Ashmaig AA, Hasan, El-Gaali E. Identification of lactic acid bacteria isolated from traditional Sudanese fermenred camel's milk (Gariss). Afr J Microbiol Res. 2009;3:451–457.

Ashmaig A, Hasan A, El-Gaali E. Identificatino of lactic acid bacteria isolated from traditional Sudanese fermented camel's milk (Gariss). Afr J Microbiol Res. 2009;3:451–7.

Aymerich T, Martı′n B, Garriga M, Vidal-CarouM.C, Bover-Cid S, Hugas M. Safety properties and molecular strain typing of lactic acidnbacteria from slightly fermented sausages. J Appl Microbiol. 2006;100:40–49.

Azar M, Ter-Sarkissian N, Ghavifek H, Ferguson T, Ghassemi H. Microbiological aspects of Sangak bread. Int J Food Sci Technol. 1977;14:251–4.

Bao QH, Chen X, Liu HX, Zhang WY, Liu WJ, Yu J, Wang F, Zhang HP. Isolation and identification of cultivable lactic acid bacteria from traditional goat milk cake in Yunnan province of China. Afr J Microbiol Res. 2011;5(29):5284–91.

Bao QH, Yu J, Liu WJ, Qing MJ, Wang WH, Xia Chen Wang F, Li MH, Wang HM Lv Q, Zhang HP. Predominant lactic acid bacteria in traditional fermented yak milk products in the Sichuan Province of China. Dairy Sci Technol. 2012a;92:309–319.

Bao QH, Liu WJ, Yu J, Wang WH, Qing MJ, Chen X, Wang F, Zhang JC, Zhang WY, Qiao JM, Sun TS, Zhang HP. Isolation and identifi cation of cultivable lactic acid bacteria in traditional yak milk products of Gansu Province in China. J Gen Appl Microbiol. 2012b;58:95–105.

Barber S, Ba′guena R, Martinez-Anaya MA, Torner MJ. Microflora de la masa madre panaria. I. Identificacion propiedades funcionales de microorganismos de masas madre industriales, elaboradas con harina de trigo. Revista de Agroquı′mica y Tecnologia de Alimentos. 1983;23:552–562.

Batt CA. *Lactococcus* introduction. In: Robinson RK, Batt CA, Patel PD, editors. Encyclopedia of food microbiology. San Diego: Academic Press; 2000. P. 1164–1166.

Boraam F, Faid M, Larpent JP, Breton A. Lactic acid bacteria and yeasts associated with traditional sourdough Moroccan bread. Sciences des Aliments. 1993;13:501–9.

Bouton Y, Guyot P, Beuvier E, Tailliez P, Grappin R. Use of PCR-based methods and PFGE for typing and monitoring homofermentative lactobacilli during Comté cheese ripening. Int J of Food Microbiol. 2002;76(1):27–38.

Bringel F, Castioni A, Olukoya DK, et al. *Lactobacillus plantarum* subsp. *argentoratensis* subsp. nov., isolated from vegetable matrices. Int J Syst Bacteriol. 2005;55(4):1629–34.

Brooijmans RJW, de Vos WM, Hugenholtz J. The electron transport chains of *Lactobacillus plantarum* WCFS1. Appl Environ Microbiol. 2009;75:3580–5.

Bui TPN, Kim YJ, In JG, Yang DC. *Lactobacillus koreensis* sp. nov., isolated from the traditional Korean food kimchi. Int J Syst Evol Microbiol. 2011;61:772–776.

Burentegusi MT, Nakamura S, et al. Identification of lactic acid bacteria isolated from fermented mare‘s milk ”chigee” in Inner Mongolia. China Anim Sci Technol. 2002;73:441–8.

Cai Y. Identification and characterization of Enterococcus species isolated from forage crops and their influence on silage fermentation. J Dairy Sci. 1999;82:2466–71.

Cai YM, Yang JS, Pang HL, Kitahara M. *Lactococcus fujiensis* sp. nov., a lactic acid bacterium isolated from vegetable matter. Int J Syst Evol Microbiol. 2011;61:1590–4.

Cai YM, Pang HL, Kitahara MK, Ohkum M. *Lactobacillus nasuensis* sp. nov., a lactic acid bacterium isolated from silage, and emended description of the genus *Lactobacillus*. Int J Syst Evol Microbiol. 2012;62:1140–4.

Callon C, Delbes C, Duthoit F, Montel MC. Application of SSCP-PCR fingerprinting to profile the yeast community in raw milk Salers cheeses. Syst Appl Microbiol. 2006;29:172–80.

Callon C, Duthoit F, Delbes C, Ferrand M, Le Frileux Y, De Cremoux R, Montel MC. Stability of microbial communities in goat milk during a lactation year: molecular approaches. Syst Appl Microbiol. 2007;30:547–60.

Camu N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama JS, Vancanneyt M, De Vuyst L. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Appl Environ Microbiol. 2007;73(6):1809–24.

Carvalho MGS, Shewmaker PL, Steigerwalt AG., Morey RE, Sampson AJ, Joyce K, Barrett TJ, Teixeira LM, Facklam RR. *Enterococcus caccae* sp. nov., isolated from human stools. Int J Syst Evol Microbiol. 2006;56:1505–1508.

Chao SH, Kudo Y, Tsai YC, Watanabe K. *Lactobacillus futsaii* sp. nov., isolated from fu-tsai and suan-tsai, traditional Taiwanese fermented mustard products. Int J Syst Evol Microbiol. 2012;62:489–494.

Chao SH, Wu RJ, Watanabe K, Chieh YT. Diversity of lactic acid bacteria in suan-tsai and fu-tsai, traditional fermented mustard products of Taiwan. Int J Food Microbiol. 2009;135:203–10.

Chaoi SH, Tomii Y, Watanabe K, Tsai YC. Diversity of lactic acid bacteria in fermented brines used to make stinky tofu. Int J Food Microbiol. 2008;123:134–41.

Chelule PK, Mokoena MP, Gqaleni N. Advantages of traditional lactic acid bacteria fermentation of food in Africa. In: Méndez-Vilas A, editor. Current research, technology and education topics in applied microbiology and microbial biotechnology. 2nd ed. Formatex Research Center; 2010. p. 1160–1167.

Chen H, Wang SY, Chen MJ. Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. Food Microbiol. 2008;(25)3:492–501.

Chen YS, Chang CH, Pan SF, Wang LT, Chang YC. *Lactococcus taiwanensis* sp. nov., a novel lactic acid bacterium isolated from fresh cummingcordia. IJSEM Papers in Press. Published November 23, 2012; doi:10.1099/ijs.0.045757-0.

Chen XP, Liu HY, Wei XC, Ma YM, Song LJ. Study on seqpration and identification of lactic acid bacteria from naturally fermented sauerkraut. Food sci China (with Englisth abstract). 2006a;27(2):91–6.

Chen YS, Yanagida F, Hsu JS. Isolation and characterization of lactic acid bacteria from suan-tsai (fermented mustard), a traditional fermented food in Taiwan. J Appl Microbiol. 2006;101:125–30.

Chen YF, Sun TS, Wang JC, Airden CK, Bai M, Zhang HP. Comparison of nutrition and microbiological compositions between two types of fermented milk from Tibet in China. Int J Food Sci Nutr. 2009;60(S7):243–50.

Chen YS, Lin YH, Pan SF, Ji SH, Chang YC, Yu CR, Liou MS, Wu HC, Otoguro M, Yanagida F, Liao CC, Chiu CM, Huang BQ. *Enterococcus saccharolyticus* subsp. *taiwanensis* subsp. nov., isolated from broccoli. Int J Syst Evol Microbiol. 2013a;63:4691–7.

Chen YS, Chang CH, Pan SF, Wang LT, Chang YC, Wu HC, Yanagida F. *Lactococcus taiwanensis* sp. nov., a lactic acid bacterium isolated from fresh cummingcordia. Int J Syst Evol Microbiol. 2013b;63:2405–9.

Cho SL, Nam SW, Yoon JH, Lee JS, Sukhoom A, Kim W. *Lactococcus chungangensis* sp. nov., a lactic acid bacterium isolated from activated sludge foam. Int J Syst Evol Microbiol. 2008;58:1844–9.

Choi HJ, Cheigh CI, Kim SB, Lee JC, Lee DW, Choi SW, Park JM, Pyun YR. Weissella kimchii sp. nov., a novel lactic acid bacterium from kimchi. Int J Syst Evol Microbiol. 2002;52:507–11.

Clementine KA, Nguessan KF, Thomas DA, Dje MK, Montet D. Application of culture dependent methods and culture-independent methods (DGGE analysis) to study Lactic acid bacteria ecology of Ivorian fermented fish Adjuevan. Chall Mod Technol. 2012;3(1):51–6.

Cocolin L, Aggio D, Manzano M, Cantoni C, Comi G. An application of PCR-DGGE analysis to profile the yeast populations in raw milk. Int Dairy J. 2002;12:407–11.

Cocolin L, Rantsiou K, Iacumin L, Urso R, Cantoni C, Comi G. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Appl Environ Microbiol. 2004;70(4):1883–94.

Collins MD, Jones D, Farrow JAE, Kilpper-Balz R, Schleifer KH. *Enterococcus avium* nom. rev., comb. nov.; *E. casselipavus* norn. rev., comb. nov.; *E. durans* norn. rev., comb. nov.; *E. gallinarum* comb. nov.; and *E. malodoratus* sp. Nov. Int J Syst Evol Bacteriol. 1984;34(2):220–223.

Collins MD, Farrow JAE, Jones D. *Enterococcus mundtii* sp. nov. Int J Syst Bacteriol. 1986;36:8–12.

Collins MD, Farrow JAE, Phillips BA, Ferusu S, Jones D. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, Carnobacterium. Int J Syst Bacteriol. 1987;37:310–6.

Collins MD, Facklam RR, Farrow JAE, Williamson R. *Enterococcus raffinosus* sp. nov., *Enterococcus solitarius* sp. nov. and *Enterococcus pseudoavium* sp. nov. FEMS Microbiol Lett. 1989;57:283–8.

Collins MD, Rodrigues UM, Pigott NE, Facklam RR. Validation List no. 38 Int J Syst Bacteriol. 1991;41:456–457.

Collins MD, Rodrigues UM, Ash C, Aguirre M, Farrow JAE, Martinez-Murcia A, Phillips BA, Williams AM, Wallbanks S. Phylogenetic analysis of the genus Lactobacillus and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol Lett. 1991b;77:5–12. doi:10.1111/j.1574-6968.1991.tb04313.x.

Collins MD, Samelis J, Metaxopoulos J, Wallbanks S. Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus Weissella for the *Leuconostoc paramesenteroides* group of species. J Appl Microbiol. 1993a;75:595–603.

Collins MD, Metaxopoulos J, Wallbanks S. Taxonomic study on some leuconostoc-like organisms from fermented sausages: Description of a new genus Weissella for the Leuconostoc paramesenteroides group of species. J Appl Bacteriol. 1993b;75:595–603.

Collins MD, Jovita RM, Hutson RA, Ohlén M, Falsen E. *Aerococcus christensenii* sp. nov., from the human vagina. Int J Syst Evol Microbiol. 1999;49:1125–8.

Coppola S, Pepe O, Masi P, Sepe M. Characterization of leavened doughs for pizza in Naples. Adv Food Sci. 1996;18:160–2.

Coppola R, Blaiotta G, Ercolini D, Moschetti G. Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese. J Appl Microbiol. 2001;90:414–20.

Corsetti A, Lavermicocca P, Morea M, Baruzzi F, Tosti N, Gobbetti M. Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species Triticum durum and Triticum aestivum) sourdoughs of Southern Italy. Int J Food Microbiol. 2001;64:95–104.

Cotta MA, Whitehead TR, Falsen E, Moore E, Lawson PA. Erratum to: Two novel species *Enterococcus lemanii* sp. nov. and *Enterococcus eurekensis* sp. nov., isolated from a swine-manure storage pit. Antonie Van Leeuwenhoek. 2013;103:1409–18.

Cousin S, Gulat-Okalla ML, Motreff L, Gouyette C, Bouchier C, Dominique Clermont, Bizet C. *Lactobacillus gigeriorum* sp. nov., isolated from chicken crop. Int J Syst Evol Microbiol. 2012;62:330–334.

Cousin S, Motreff L, Gulat-Okalla ML, Gouyette C, Cathrin S, Schumann P, Begaud E, Bouchier C, Clermont D, Bizet C. *Lactobacillus pasteurii* sp. nov. and *Lactobacillus hominis* sp. nov. Int J Syst Evol Microbiol. 2013;63:53–9.

David B, Dennis SN, Kim IS Finn KV, Sawadogo-Lingania H, Derkx PMF, Jespersena L. *Lactobacillus delbrueckii* subsp. *jakobsenii* subsp. nov., isolated from dolo wort; an alcoholic fermented beverage in Burkina Faso Int J Syst Evol Microbiol. 2013. doi:10.1099/ijs.0.048769-0.

De graef EM, Devriese LA, Vancanneyt M., Baele M, Collins MD, Lefebvre K, Swings J, Haesebrouck F. Description of *Enterococcus canis* sp. nov. from dogs and reclassification of *Enterococcus porcinus* (Teixeira et al. 2001 as a later synonym of *Enterococcus villorum* Vancanneyt et al. 2001). Int J Syst Evol Microbiol. 2003;53:1069–1074.

De vaux A, Laguerre G., Diviès C, Prévost H. *Enterococcus asini* sp. nov. Isolated from the caecum of donkeys (Equus asinus). Int J Syst Evol Bacteriol. 1998;48:383–387.

Delbes C, Ali-Mandjee L, Montel MC. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. Appl Environ Microbiol. 2007;73:1882–91.

Dessart SR, Steenson LR. Biotechnology of dairy Leuconostoc. In: Hui YH, Khachatourians GG, editors. Food biotechnology: microorganisms. New York: Wiley-Interscience; 1995. p. 665–702.

Devriese LA, Ceyssens K, Rodrigues UM, Collins MD. *Enterococcus columbae*, a species from pigeon intestines. FEMS Microbiol Lett. 1990;71:247–52.

Devriese LA, Pot B, Collins MD. Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species group. J Appl Bacteriol. 1993;75:399–408.

Dewan S, Tamang JP.Dominant lactic acid bacteria and their technological properties isolated from the Himalayan ethnic fermented milk products. Antonie van Leeuwenhoek. 2007;92:343–352.

Di Cagno R, Tamborrino A, Gallo G, Leone C, de Angelis M, Faccia M, Amirante P, Gobbetti M. Uses of mares' milk in manufacture of fermented milks. Int Dairy J. 2004;14:767–75.

Dicks LMT, Fantuzzi L, Gonzalez FC, Du toit M, Dellaglio F. *Leuconostoc argentinum* sp. nov., isolated from argentine raw milk. Int J Syst Bacteriol. 1993;43:347–351.

Dicks L, Dellaglio F, Collins M. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. Int J Syst Evol Microbiol. 1995;45:395–7.

Dirar HA. The indigenous fermented foods of the sudan. A study in African food and Nutrition, CAB International, Cambridge, UK. 1993.

Dmitriy VV, Amselle M, Beck BJ, Popham D L, Whittaker P, Wang H, Kerrigan E, Chizhikov VE. *Lactobacillus brantae* sp. nov., isolated from faeces of Canada geese (Branta canadensis). J Syst Evol Microbiol. 2012;62:2068–2076.

Dobson A, O'sullivan O, Cotter PD, Ross P, Hill C. High-throughput sequence-based analysis of the bacterial composition of kefir and an associated kefir grain. FEMS Microbiol Lett. 2011;320:56–62.

Doi K, Nishizaki Y, Fujino Y, Ohshima T, Ohmomo S, Ogata S. *Pediococcus lolii* sp. nov., isolated from ryegrass silage. Int J Syst Evol Microbiol. 2009;59:1007–1010.

Dolci P, Alessandria V, Rantsiou K, Rolle L, Zeppa G, Cocolin L. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. Int J Food Microbiol. 2008;122:302–11.

Dolci P, Alessandria V, Rantsiou K, Bertolino M, Cocolin L. Microbial diversity, dynamics and activity throughout manufacturing and ripening of Castelmagno PDO cheese. Int J Food Microbiol. 2010;143:71–5.

Duan Y, Tan Z, Wang Y, Li Z, Li Z, Qin G, Huo Y, Cai Y. Identification and characterization of lactic acid bacteria isolated from Tibetan Qula cheese. J Gen Appl Microbiol. 2008;54:51–60.

Duthoit F, Godon JJ, Montel M. Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. Appl Environ Microbiol. 2003;69:3840–8.

El Mardi MM. A study on fermented milk 'roub'. M.Sc. thesis, University of Khartoum, Sudan. 1988.

El-Baradei G, Delacroix-Buchet A, Jean-Claude O. Biodiversity of bacterial ecosystems in traditional Egyptian Domiati Cheese. Appl Environ Microbiol. 2007;73(4):1248–1255.

El-Owni AOO, Hamid OIA. Effect of storage period on weight loss, chemical composition, microbiological and sensory characteristics of Sudanese white cheese (Gibna Bayda). Pak J Nutr. 2008;7:75–80.

EI-Sheikh NAA. Production of Mudaffara cheese from cows and goats milk. M.Sc. Thesis, University of Khartoum, Sudan. 1997.

Elisa S, Sandra T, Felis GE. The Genus *Lactobacillus*: a taxonomic update. Probiotics Antimicro Prot. 2012;4:217–226.

Elmagli AAO, El-Zubeir IEM. Study on the compositional quality of pasteurized milk in Khartoum state (Sudan). Int J Dairy Sci. 2006;1:12–20.

Endo A, Irisawa T, Futagawa-Endo Y, Takano K, MT, Okada S, Dicks L M. T. Characterization and emended description of *Lactobacillus kunkeei* as a fructophilic lactic acid bacterium. Int J Syst Evol Microbiol. 2012;62:500–504.

Endo A, Okada S. Reclassification of the genus Leuconostoc and proposals of *Fructobacillus fructosus* gen. nov., comb. nov., *Fructobacillus durionis* comb. nov., *Fructobacillus ficulneus* comb. nov. and *Fructobacillus pseudoficulneus* comb. nov. Int J Syst Evol Microbiol. 2008;58:2195–205.

Endo A, Futagawa-Endo Y, Schumann P, Pukall R, Dicks LMT. *Bifidobacterium reuteri* sp. nov., *Bifidobacterium callitrichos* sp. nov., *Bifidobacterium saguini* sp. nov., *Bifidobacterium stellenboschense* sp. nov. and *Bifidobacterium biavatii* sp. nov. isolated from faeces of common marmoset (Callithrix jacchus) and red-handed tamarin (Saguinus midas). Syst Appl Microbiol. 2012;35:92–7.

Engelen B, Meinken K, von Wintzingerode F, Heuer H, Malkomes HP, Backhaus H. Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic engerprinting in addition to conventional testing procedures. Appl Environ Microbiol. 1998;64:2814–21.

Ercolini D, Mauriello G, Blaiotta G, Moschetti G, Coppola S. PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarella cheese. J Appl Microbiol. 2004;96:263–70.

Escalante A, Wacher C, Farres A. Lactic acid bacterial diversity in the traditional Mexican fermented dough pozol as determined by 16S rDNA sequence analysis. Int J Food Microbiol. 2001;64:21–31.

Euzeby JP. List of prokaryotic names with standing in nomenclature—genus leuconostoc. http://www.bacterio.cict.fr/l/leuconostoc.html (2009). Accessed 02/05 2010.

Facklam RR, Elliot JA. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. Clin Microbiol Rev. 1995;8:470–95.

Faid M, Boraam F, Zyani I, Larpent JP. Characterization of sourdough bread ferments made in the laboratory by traditional methods. Zeitschrift für Lebensmittel Untersuchung und Forschung. 1994;198:287–91.

Farah Z, Mollet M, Younan M, Dahir R. Camel dairy in Somalia: limiting factors and development potential. Livest Sci. 2007;110:187–91.

Farrow JAE, Collins MD. *Enterococcus hirae*, a new species that includes amino acid assay strain NCDO 1258 and strains causing growth depression in young chickens. Int J Syst Bacteriol. 1985;35:73–5.

Farrow JAE, Facklam RR, Collins MD. Nucleic acid homologies of some vancomycin-resistant leuconostocs and description of *Leuconostoc citreum* sp. nov. and Leuconostoc pseudomesenteroides sp. nov. Int J Syst Bacteriol. 1989;39:279–83.

Felis GE, Dellaglio F. Taxonomy of lactobacilli and bifidobacteria. Curr Issues Intest Microbiol. 2007;8:44–61.

Felis GE, Torriani S, Dellaglio F. Reclassification of *Pediococcus urinaeequi* (ex Mees 1934) Garvie 1988 as *Aerococcus urinaeequi* comb. nov. Int J Syst Evol Bacteriol. 2005;55:1325–1327.

Fennema OF, Hui YH, Karel M, Walstra P, Whitaker JR. Lactic acid bacteria (Microbiological and Functional Aspects) In: Salminen S, von Wright A, editors. Food science and technology a series of monographs, textbooks, and reference books. 3rd ed. New York: Marcel Dekker, Inc.; 2004. P. 19–30.

Feresu SB, Muzondo MI. Identification of some lactic acid bacteria from two Zimbabwean fermented milk products. World J Microbiol Biotechnol. 1990;6:178–86.

Feurer C, Irlinger F, Spinnler HE, Glaser P, Vallaeys T. Assessment of the rind microbial diversity in a farmhouse-produced vs a pasteurized industrially produced soft red-smear cheese using both cultivation and rDNA-based methods. J Appl Microbiol. 2004;97:546–56.

Forsum U, Holst E, Larsson PG, Vasquez A, Jakobsson T, Mattsby-Baltzer I. Bacterial vaginosis—a microbiological and immunological enigma. Minirev APMIS. 2005;113:81–90.

Fortina MG, Ricci G, Mora D, Manachini PL. Molecular analysis of artisanal Italian cheeses reveals *Enterococcus italicus* sp. nov. Int J Syst Evol Bacteriol. 2004;54:1717–1721.

Frolkova′P, Pavel Švec, Sedláček I, Mašlaňova′I, Černohla′vkova′J, Ghosh A, Zurek L, Radimersky T and Literák I. *Enterococcus alcedinis* sp. nov., isolated from common kingfisher (Alcedo atthis). Int J Syst Evol Microbiol. 2013;63:3069–3074.

Gadaga TH, Mutukumira AN, Narvhus JA. Growth characteristics of Candida kefir and two strains of *Lactococcus lactis* subsp. *lactis* isolated from Zimbabwean naturally fermented milk. Int J Food Microbiol. 2001;70(1–2):11–9.

Gagnaire V, Thierry A, L′eonil J. Propionibacteria and facultatively heterofermentative lactobacilli weakly contribute to secondary proteolysis of Emmental cheese. Lait. 2001;81:339–353.

Gala E, Landi S, Solieri L, Nocetti M, Pulvirenti A, Giudici P. Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. Int J Food Microbiol. 2008;125:347–51.

Galli A, Franzetti L, Fortina MG. Isolation and identification of sour dough microflora. Microbiologie–Aliments–Nutrition. 1988;6:345–351.

Galli A, Ottogalli G. Aspetti della microflora degli impasti panettone. Annali di Microbiologia e Enzimologia. 1973;23:39–49.

Garrity GM, Bell JA, Lilburn TG. Taxonomic outline of the procaryotes. Bergey's manual of systematic bacteriology, 2nd ed, Release 5.0. New York: Springer. 2004; doi:http://dx.doi.org/10.1007/bergeysoutline200405.

Garvie EI. Hybridization between deoxyribonucleic acids of some strains of heteroermentative lactic acid bacteria. Int J Syst Bacteriol. 1976;26:116–22.

Garvie EI. *Pediococcus urinaeequi* nom. rev. In validation of the publication of new names and combinations previously effectively published outside the IJSB, List no. 25. Int J Syst Bacteriol. 1988;38:220–2.

Garvie, E. Genus *Leuconostoc*. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology. Vol 2. Baltimore: The Williams & Wilkins Co.; 1986. p. 1071.

Gobbetti M, Corsetti A, Rossi J. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of amino acids. World J Microbiol Biotechnol. 1994;10:275–9.

González-Arenzana L, López R, Santamaría P, López-Alfaro I. Dynamics of lactic acid bacteria populations in Rioja wines by PCR-DGGE, comparison with culture-dependent methods. Appl Microbiol Biotechnol. 2013;97(15):6931–41.

Goodfellow M. Phylum XXVI. Actinobacteria phyl. nov. In: Goodfellow M, Kämpfer P, Busse HJ, Trujillo M, Suzuki KI, Ludwig W et al., editors. Bergey's manual of systematic bacteriology. New York: Springer; 2012. p. 171–188.

Gu CT, Li CY, Yang LJ, Huo GC. *Lactobacillus heilongjiangensis* sp. nov., isolated from Chinese pickle. Int J Syst Evol Microbiol. 2013;63:4094–4099.

Gu CT, Wang F, Li CY, Liu F, Cheng HG. *Lactobacillus xiangfangensis* sp. nov., isolated from Chinese pickle. Int J Syst Evol Microbiol; 2012;62:860–863.

Hamad SH, Dieng MC, Ehrmann MA, Vogel RF. Characterization of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. J Appl Microbiol. 1997;83:764–70.

Hamid IA, AO El Owni. Microbiological properties and sensory characteristics of white cheese (Gibna bayda) collected in Zalengei area West Darfur. Res J Anim Vet Sci 2007;2:61–5.

Hammes WP. Starterkulturpriparate in der Fleishwirtschaft. Cherm Mikrobiol. Technol Lebensmittel. 1985;9:131–43.

Hammes WP, Hertel C. Genus I. *Lactobacillus* Beijerink 1901 In: De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB, editors. Bergey's manual of systematic bacteriology, vol. 3, 2nd ed. Berlin: Springer; 2009. p. 465–510.

Hammes WP. Annegret Banfleon and Seunghwa Min. Lactic acid bacteria in meat fermentation. FEMS Microbiol Rev. 1990;87:165–174.

Hammes WP, Hertel C. The Genera *Lactobacillus* and *Carnobacterium*. In: Dwork M, editor. The Prokaryotes Release 3.15. 2003. p. 320–440.

Hammes WP, Vogel RF. The genus Lactobacillus. In: Wood BJB, Holzapfel WH, editors. The genera of lactic acid bacteria. London: Blackie Academic & Professional; 1995. p. 19–54.

Hamza AA, El Gaali IE, Mahdi AA. Use of the RAPD-PCR fingerprinting and API system for clustering lactic acid bacteria isolated from traditional Sudanese sour milk (Roab). Afr J Biotechnol. 2009;8:3399–404.

Hassan AR, El–Zubeir MEI, Babiker AS. Chemical and microbial measurements of fermented camel milk Garris from transhumant and nomadic herds in Sudan. J Basic Appl Sci. 2008;2:166–171.

Henri-Dubernet S, Desmasures N, Gueguen M. Diversity and dynamics of *lactobacilli* populations during ripening of RDO Camembert cheese. Canad J Microbiol. 2008;54:218–28.

Hill GB, Eschenbach DA, Holmes KK. Bacteriology of the vagina. Scand J Urol Nephrol. 1984;86:23–39.

Hiu SF. Lactobacillus piscicola, a new species from salmonid fish. Int J Syst Evol Bacteriol. 1984;34:393–400.

Holzapfel WH, Gerber ES. *Lactobacillus divergens*. sp. nov. a new heterofermentative *Lactobacillus* species producing L(+)-lactate. Syst Appl Microbial. 1983;4:522–534.

Horvath P, Coûté-Monvoisin AC, Romero DA, Boyaval P, Fremaux C, Barrangou R. Comparative analysis of CRISPR loci in lactic acid bacteria genomes. Int J Food Microbiol. 2009;131:62–70.

Hugenholtz P, Pitulle C, Hershberger KL, Pace NR. Novel division level bacterial diversity in a Yellowstone hot spring. J Bacteriol. 1998;180:366–76.

Hui YH, Evarnuz Ö, Noé Arroyo-López F. Handbook of plant-pased fermented food and beverage technology. Boca Raton: CRC Press; 2012. p. 58–61.

Humblot C, Guyot JP. Pyrosequencing of tagged 16S rRNA gene amplicons for rapid deciphering of the microbiomes of fermented foods such as pearl millet slurries. Appl Environ Microbiol. 2009;75:4354–61.

Infantes M, Tourneur C. Etude de la flore lactique de levains naturels de panification provenant de diffe′rentes re′gions françaises. Sciences des Aliments. 1991;11:527–45.

Jay JM. Modern food microbiology 5th edition. New York: Chapman & Hall, 1996; p. 137–41, 328–42, 347–52.

Jiri MT, Cui P, Ding F, Geng J, Gao H, Zhang H, Yu J, Hu S, Meng H. Monophyletic origin of domestic bactrian camel (*Camelus bactrianus*) and its evolutionary relationship with the extant wild camel (Camelus bactrianus ferus). Anim Genet. 2009;40:377–82.

Jones D. Composition and differentiation of the genus *Streptococcus*. In Streptococci. 1978; p. 1–49.

Jung JY, Lee SH, Kim JM, Park MS, Bae JW, Hahn Y, Madsen EL, Jeon CO. Metagenomic analysis of kimchi, a traditional Korean fermented food. Appl Environ Microbiol. 2011;77:2264–74.

Kandler O. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek. 1983;49:209–24.

Kandler O, Weiss N. The genus *Lactobacillus*. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. Bergey's manual of systematic bacteriology. Vol 2. Baltimore: Williams & Wilkins; 1986. P. 1209–1235, 1208–1234.

Kazanskaya LN, Afanasyeva OV, Patt VA. Microflora of rye sours and some specific features of its accumulation in bread baking plants of the USSR. In Holas J, Kratochvil F editors. Developments in food science. Progress in cereal chemistry and technology. Vol 5B. London: Elsevier; 1983. p. 759–763.

Kebede A, Viljoen BC, Gadaga H, Narvhus JA, Analie LH. The effect of incubation temperature on the survival and growth of yeasts in Sethemi, South African naturally fermented milk. Food Technol Biotechnol. 2007;45(1):21–6.

Keller JJ, Jordan I. Fermented milks for the South African market. Afric J Dairy Sci. 1990;22:47–9.

Kesmena Z, Yetimana AE, Gulluce A, Kacmazc N, Sagdicd O, Cetine B, Adiguzelf A, Sahing F, Yetima H. Combination of culture-dependent and culture-independent molecular methods for the determination of lactic microbiota in sucuk. Int J Food Microbiol. 2012;53(3):428–435.

Killer J, Kopečný J, Mrázek J, Koppová I, Havlík J, Benada O, kott T. *Bifidobacterium actinocoloniiforme* sp. nov. and *Bifidobacterium bohemicum* sp. nov., from the bumblebee digestive tract. Int J Syst Evol Microbiol. 2011;61:1315–1321.

Kim M-S, Seong WR, Nam YD, Yoon JH, Bae JW. *Carnobacterium jeotgali* sp. nov., isolated from a Korean traditional fermented food. Int J Syst Evol Bacteriol. 2009;59:3168–3171.

Kim TW, Lee JY, Jung SH, Kim YM, Jo JS, Chung DH, Lee HJ, Kim HY. Identification and distribution of predominant lactic acid bacteria in kimchi; a Korean traditional fermented food. J Micribiol Biotechnol. 2002;12:635–42.

Kim B, Lee J, Jang J, Kim J, Han H. *Leuconostoc inhae* sp. nov., a lactic acid bacterium isolated from kimchi. Int J Syst Evol Microbiol. 2003;53:1123–6.

Kim J, Kim JY, Kim MS, Roh SW, Bae JW. *Lactobacillus kimchiensis* sp. nov., isolated from a fermented food. Int J Syst Evol Microbiol. 2013;63:1355–9.

Klaenhammer TR, Altermann E, Arigoni F, Bolotin A, Breidt F, Broadbent J, Cano R, Chaillou S, Deutscher J, Gasson M, van de Guchte M, Guzzo J, Hartke A, Hawkins T, Hols P, Hutkins R, Kleerebezem M, Kok J, Kuipers O, Lubbers M, Maguin E, McKay L, Mills D, Nauta A, Overbeek R, Pel H, Pridmore D, Saier M, van Sinderen D, Sorokin A, Steele J, O'Sullivan D, de Vos W, Weimer B, Zagorec M, Siezen R. Discovering lactic acid bacteria by genomics. Antonie Van Leeuwenhoek. 2002;82:29–58.

Klaenhammer TR, Barrangou R, Buck BL, Azcarate-Peril MA, Altermann E. Genomic features of lactic acid bacteria effecting bioprocessing and health. FEMS Microbiol Rev. 2005;29:393–409.

Kleerebezem M, Hugenholtz J. Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol. 2003;14:232–7.

Klein G, Alexander P, Christine B, Gerhard R. Taxonomy and physiology of probiotic lactic acid bacteria. Int J Food Microbiol. 1998;41:103–25.

Kline L, Sugihara TF. Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. Appl Microbiol. 1971;21:459–65.

Konigs WN, Kok J, Kuipers OP, Poolman B. Lactic acid bacteria: the bug of the new millennium. Curr Opin Microbiol. 2000;3:276–82.

Koort J, Coenye T, Vandamme P, Sukura A, Björkroth J. *Enterococcus hermanniensis* sp. nov., from modified-atmosphere-packaged broiler meat and canine tonsils. Int J Syst Evol Microbiol. 2004;54:1823–7.

Kostinek M, Specht I, Edward VA, Schillinger U, Hertel C, Holzapfela WH, Franz CMAP. Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of Gari, a traditional African food. Syst Appl Microbiol. 2005;28:527–40.

Kudo Y, Oki K, Watanabe K. *Lactobacillus delbrueckii* subsp. *sunkii* subsp. nov. isolated from sunki, a traditional Japanese pickle. Int J Syst Evol Microbiol. 2012;62:2643–9.

Kurmann JA, Rasic JLJ, Kroger M. Encyclopedia of fermented. Fresh milk products. New York: Van Nostrand Reinhold; 1992.

Kusuda R, Kawai K, Salati F, Banner CR, Fryer JL. *Enterococcus seriolicida* sp. nov., a fish pathogen. Int J Syst Bacteriol. 1991;41:406–9.

Lafarge V, Ogier JC, Girard V, Maladen V, Leveau JY, Gruss A, Delacroix-Buchet A. Raw cow milk bacterial population shifts attributable to refrigeration. Appl Environ Microbiol. 2004;70:5644–5650.

Lahtinen S, Salminen S, Ouwehand A, Wright AV. Lactic acid bacteria, Microbiological and functional aspects. 4th ed. Boca Raton: CRC Press; 2011.

Lancefield RC. A serological differentiation of human and other groups of hemolytic streptococci. J Exp Med. 1933;57(4):571–95. doi:10.1084/jem.57.4.571. PMC 2132252. PMID 19870148.

Lattanzi A, Minervini F, Di Cagno R, Diviccaro A, Antonielli L, Cardinali G, Cappelle S, De Angelis M, Gobbetti M. The lactic acid bacteria and yeastmicrobiota of eighteen sourdoughs used for the manufacture of traditional Italian sweet leavened baked goods. Int J Food Microbiol. 2013;163:71–9.

Law-Brown J, Meyers PR. Enterococcus phoeniculicola sp. nov., a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe, Phoeniculus purpureus. Int J Syst Evol Microbiol. 2003;53:683–5.

Lawson PA, Falsen E, Ohlén M, Collins MD. *Aerococcus urinaehominis* sp. nov., isolated from human urine. Int J Syst Evol Bacteriol. 2001a;51:683–686.

Lawson PA, Falsen E, Truberg-Jensen K, Collins MD. *Aerococcus sanguicola* sp. nov., isolated from a human clinical source. Int J Syst Evol Bacteriol. 2001b;51:475–479.

Lee HJ, Park CH, Joo YJ, Kim SH, Yoon JH, Park YH, Hwang IK, Ahn JS, Mheen TI. Identification and characterization of bacteriocin-producing lactic acid bacteria isolated from kimchi. J Microbiol Biotechnol. 1999;9:282–91.

Lee JS, Lee KC, Ahn JS, Mheen TI, Pyun YR, Park YH. *Weissella koreensis* sp. nov., isolated from kimchi. Int J Syst Evol Microbiol. 2002;52:1257–61.

Lei Xiao, Sun Guipeng, Xie Jingli, Wei Dongzhi. *Lactobacillus curieae* sp. nov., isolated from stinky tofu brine. Int J Syst Evol Microbiol. 2013;63:2501–5.

Leisner JJ, Vancanneyt M, Van Der Meulen R, Lefebvre K, Engelbeen K, Hoste B, Laursen BG, Bay L, Rusul G, De Vuyst L, Swings J. *Leuconostoc durionis* sp. nov., a heterofermenter with no detectable gas production from glucose. Int J Syst Evol Microbiol. 2005;55:1267–70.

Leisner JJ, Laursen BG, Prevost H, Drider D, Dalgaard P. Carnobacterium: positive and negative effects in the environment and in foods. FEMS Microbiol Rev. 2007;31:592–613.

Lerche M. Die deulschen Wurslerzeuipliue. Eine Systtematik der Wurstarten Wursterzeugnisse in der Bundesrepublik und in Westberlin. Arbeium der DLG. Band 134 DLG-Verlag Frankfurt a. M; 1975.

Li W, Shang H, Li J, Xu Z, Qin L. The content of Microorganism of yak's milk of Tianjin regions. Chin J Anim Quaran. 2002;19:36.

Liang ZQ, Sathiyaraj Srinivasan et al. *Lactobacillus kimchicus* sp. nov., a β-glucosidase-producing bacterium isolated from kimchi. Int J Syst Bacteriol. 2011;61:894–897.

Lin C, Bolsen KK, Brent BE, Fung DYC. Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize. J Appl Bacteriol. 1992;73:375–87.

Liu WJ, Sun ZH, Zhang JC, Gao W, Wang WH, Wu L, Sun TS, Chen W, Liu XM, Zhang HP. Analysis of microbial composition in acid whey for dairy fan making in Yunnan by conventional method and 16S rRNA sequencing. Curr Microbiol. 2009;59:199–205.

Lönner C, Welander T, Molin N, Dosta′lek M, Blickstad E. The microflora in a sour dough started spontaneously on typical Swedish rye meal. Food Microbiol. 1986;3:3–12.

Luo Z, Li SYY, Han B. Research on the microflora and physicochemical index of the kurut in Tibet. China Brewing (in Chinese). 2005;10:40–1.

Mann EJ. Kefi r and koumiss. Dairy Ind Int. 1989;54(9):9–10.

Martinez-Murcia AJ, Collins MD. A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol Lett. 1990;70:73–83.

Martinez-Murcia AJ, Collins MD. A phylogenetic analysis of an atypical leuconostoc: description of *Leuconostoc fallax* sp. nov. FEMS Microbiol Lett. 1991a;82:55–60.

Martinez-Murcia AJ, Collins MD. Enterococcus sulfureus, a new yellow-pigmented Enterococcus species. FEMS Microbiol Lett. 1991b;80:69–74.

Martinez-Murcia AJ, Harland NM, Collins MD. Phylogenetic analysis of some *leuconostocs* and related organisms as determined from large-subunit rRNA gene sequences: assessment of congruence of smalland large-subunit rRNA derived trees. J Appl Microbiol. 1993;74:532–41.

Mathara JM, Schillinger U, Kutima PM, Mbugua SK, Holzapfel WH. Isolation, identification and characterisation of the dominant microorganisms ofkule naoto: the Maasai traditional fermented milk in Kenya. Int J Food Microbiol. 2004;94:269–78.

Mathara JM, Schillinger U, Guigas C, Franz C, Kutima PM, Mbugua SK, Shin HK, Holzapfel WH. Functional characteristics of *Lactobacillus* spp. from traditional Maasai fermented milk products in Kenya. Int J Food Microbiol. 2008;126(1–2):57–64.

Menghebilige, Wu RN, Wang LP Yang XJ, Xu J, Dong Y, Sun ZH, Zhang HP. Isolation and identification of lactobacillus from koumiss e ollected in Inner Mong olia and people 's Republic of Mongolia (Chinese with English abstract). China Dairy Ind. 2004;32(11):6–11.

Merk K, Borelli C, Korting HC. Lactobacilli—bacteria host interactions with special regard to the urogenital tract. Int J Med Microbiol. 2005;295:9–18.

Meroth CB, Walter J, Hertel C, Brandt M, Hammes WP. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol. 2003;69:475–82.

Mheen TI, Kwon TW. Effect of temperature and salt concentration on kimchi fermentation. Korean J Foo Sci Technol. 1984;16:443–50.

Rosenbach FJ. Mikro-Organismen bei den Wund-bfektions-Krankheiten des Menschen JF Bergman, Wiesbaden; 1884.

Miller A III, Morgan ME, Libbey LM. *Lactobacillus malraromicus*, a new species producing a malty aroma. Int J Syst Bacterial. 1974;24:346–54.

Minervini F, Di Cagno R, Lattanzi A, De Angelis M, Antonielli L, Cardinali G, Cappelle S, Gobbetti M. Lactic acid bacterium and yeast microbiotas of 19 sourdoughs used for traditional/typical Italian breads: interactions between ingredients and microbial species diversity. Appl Environ Microbiol. 2012;78:1251–64.

Miyazaki K, Matsuzaki T. Health Properties of Milk Fermented with *Lactobacillus casei* strain Shirota (LcS). In: Farnworth ER, editor. Handbook of fermented functional foods. 2nd ed. New York: CRC Press, Taylor & Francis Group; 2008. p. 165–208.

Montanari G, Grazia L. Galactose-fermenting yeasts as fermentation microorganisms in traditional koumiss. Food Technol Biotech. 1997;35:305–8.

Montanari G, Zambonelli C, Grazia L, Kamesheva GK, Shigaeva MK. *Saccharomyces unisporus* as the principal alcoholic fermentation microorganism of traditional koumiss. J Dairy Res. 1996;63:327–31.

Morandi S, Cremonesi P, Povolo M, Brasca M. *Enterococcus lactis* sp. nov., from Italian raw milk cheeses. Int J Syst Evol Bacteriol. 2012;62:1992–1996.

Morea M, Baruzzi F, Cocconcelli PS. Molecular and physiological characterization of dominant bacterial populations in traditional Mozzarella cheese processing. J Appl Microbiol. 1999;87:574–582.

Morita H, Nakano A, Onoda H, Toh H, Oshima K, Takami H, Murakami M, Fukuda S, Takizawa T, Kuwahara T, Ohno H, Tanabe S, Hattori M. *Bifidobacterium kashiwanohense* sp. nov., isolated from healthy infant faeces. Int J Syst Evol Microbiol. 2011;61:2610–5.

Müller, M. R. A., Wolfrum, G., Stolz, P., Ehrmann, M. A., & Vogel, R. F. Monitoring the growth of *Lactobacillus* species during arye flour fermentation. Food Microbiol. 2001;18:217–227.

Muck RE. Effects of inoculation level on alfalfa silage quality. Trans ASAE. 1989;32:1153–8.

Mundt JO. Lactic acid bacteria associated with raw plant food material. J Milk Food Technol. 1970;33:550–3.

Naser SM, Vancanneyt M, De graef E, Devriese LA, Snauwaert C, Lefebvre K, Hoste B, Švec P, Decostere A, Haesebrouck F, Swings J. *Enterococcus canintestini* sp. nov., from faecal samples of healthy dogs. Int J Syst Evol Microbiol. 2005;55:2177–2182.

Nguyen DTL, Cnockaert M, Hoorde KV, Brandt ED, Snauwaert I, Snauwaert C, Vuyst LD, Le BT, Vandamme P. *Lactobacillus porcinae* sp. nov., isolated from traditional Vietnamese nem chua. Int J Syst Evol Microbiol. 2013;63:1754–9.

Niemi RM, Ollinkangas T, Paulin L, Š vec P, Vandamme P, Karkman Antti, Kosina Marcel, Lindström K. *Enterococcus rivorum* sp. nov., from water of pristine brooks. Int J Syst Evol Microbiol. 2012;62:2169–2173.

Nikolic M, Terzic-Vidojevic A, Jovcic B, Begovic J, Golic N, Topisirovic L. Characterization of lactic acid bacteria isolated from Bukuljac, a homemade goat's milk cheese. Int J Food Microbiol. 2008;122:162–70.

Odunfa SA. African fermented Food. In: Wood BJB, editor. Microbiology of fermented food. Vol 2. Essex, England: Elsevier Applied Science Publishers Ltd. 1985;155–191.

Ogier JC, Son O, Gruss A, Tailliez P, Delacroix-Buchet A. Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Appl Environ Microbiol. 2002;68:3691–701.

Oki K, Kudo Y, Watanabe K. *Lactobacillus saniviri* sp. nov. and *Lactobacillus senioris* sp. nov., isolated from human faeces. Int J Syst Evol Microbiol. 2012;62:601–7.

Omar NB, Ampe F. Microbial community dynamics during production of the Mexican fermented maize dough pozol. Appl Environ Microbiol. 2000;66(9):3664–73.

Orla-Jensen S. The lactic acid bacteria Fred Host and Son. Copenhagen; 1919.

Ottogalli G, Galli A, Foschino R. Italian bakery products obtained with sour dough: characterization of the typical microflora. Adv Food Sci. 1996;18:131–44.

Ouadghiri M, Amar M, Vancanneyt M, Swings J. Biodiversity of lactic acid bacteria in Moroccan soft white cheese (Jben). FEMS Microbiol Lett. 2005;251:267–71.

Pahlow G, Muck RE, Driehuis F. Microbiology of ensiling. In: Silage science and technology. Madison. Proceedings… Madison: ASCSSA-SSSA, Agronomy. 2003;42:31–93.

Palomba S, Blaiotta G, Ventorino V, Saccone A, Pepe O. Microbial characterization of sourdough for sweet baked products in the Campania region (southern Italy) by a polyphasic approach. Ann Microbiol. 2011;61:307–14.

Pang H, Qin G, Tan Z, Li Z, Wang Y, Cai Y. Natural populations of lactic acid bacteria associated with silage fermentation as determined by phenotype, 16S ribosomal RNA and recA gene analysis. Syst Appl Microbiol. 2011;34:235–41.

Park W, Zhang H, Zhang B, Zhang L. Mare milk. In: Park YW, George FW, editors. Handbook of milk of non-bovine mammals. 1st ed. Haenlein: Blackwell Publishing; 2006. P. 275–296.

Park JM, Shin JH, Lee DW, Song JC, Suh HJ, Chang UJ, Kim JM. Food Sci Biotechnol. 2010;19(2):541–6.

Pederson CS. Microbiology of food fermentations. Westport: AVI; 1971.

Pikuta EV, Marsic D, Bej A, Tang J, Krader P, Hoover RB. *Carnobacterium pleistocenium* sp. nov., a novel psychrotolerant, facultative anaerobe isolated from permafrost of the Fox Tunnel in Alaska. Int J Syst Evol Bacteriol. 2005;55:473–478.

Prescott SC, Dunn CG. (1957) Industrial microbiology. New York: McGraw-Hill; 1957.

Psoni L, Tzanetakis N, Litopoulou-Tzanetaki E. Microbiological characteristics of Batzos, a traditional Greek chees from raw goat's milk. Food Microbiol. 2003;20:575–82.

Quigley L, O'Sullivan O, Beresford TP, PaulRoss R, Fitzgerald GF, Cotter PD. Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. Int J Food Microbiol. 2011;15081–5094.

Rahkila R, Johansson P, Säde E, Björkroth J. Identification of enterococci from broiler products and a broiler processing plant and description of *Enterococcus viikkiensis* sp. nov. Appl Environ Microbiol. 2011;77:1196–203.

Rahman AIE, Dirar HA, Osman MA. Microbiological and chemical changes and sensory evaluation of camel milk fermented by selected bacterial starter cultures. Afr J Food Sci. 2009;3:398–405.

Randazzo CL, Torriani S, Akkermans AD, de Vos WM, Vaughan EE. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Appl Environ Microbiol. 2002;68(4):1882–92.

Randazzo CL, Caggia C, Neviani E. Application of molecular approaches to study lactic acid bacteria in artisanal cheeses. J Microbiol Methods. 2009;78:1–9.

Rantsiou K, Urso R, Dolci P, Comi G, Cocolin L. Microflora of Feta cheese from four Greek manufacturers. Int J Food Microbiol. 2008;126:36–42.

Reuter G. Atypiaehe Streptobakteden als dominimmde flora in reifender and gelagerter Rohwurst. Fleischwirtschaft. 1967;47:397–402.

Ring E, Gatesoupe FJ. Lactic acid bacteria in fish: a review. Aquaculture. 1998;160:177–203.

Rocha JM, Malcata FX. On the microbiological profile of traditional Portuguese sourdough. J Food Prot. 1999;62:1416–29.

Roh SW, Kim KH, Nam YD, Chang HW, Park EJ, Bae JW. Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. Int J Syst Evol Microbiol. 2010;4:1–16.

Rosenquist H, Hansen A. The microbial stability of two bakery sourdoughs made from conventionally and organically grown rye. Food Microbiol. 2000;17:241–50.

Švec P, Vandamme P, Bryndova′ H, Holochova′P, Kosina M, Mašlaňová′I, Sedlácek I. *Enterococcus plantarum* sp. nov., isolated from plants. Int J Syst Evol Microbiol. 2012;62:1499–1505.

Saeed ZK. Some technological aspects of indigenous Sudanese soups (molahs). Ph.D. thesis, University of Reading, UK. 1981.

Sakamoto N, Tanaka S, Sonomoto K, Nakayama J. 16S rRNA pyrosequencing-based investigation of the bacterial community in nukadoko, a pickling bed of fermented rice bran. Int J Food Microbiol. 2011;144:352–9.

Salih AMM, El Sanousi SM, El Zubeir EM. A review on the Sudansese traditional dairy products and technology. Int J Dairy Sci. 2011;6(4):227–45.

Salovaara H, Katunpää H. An approach to the classification of Lactobacilli isolated from Finnish sour rye dough ferments. Acta Alimentaria Polonica. 1984;10:231–9.

Sawadogo-Lingani H, Diawara VLB, Nielsen DS, Møller PL, Traore AS, Jakobsen M. The biodiversity of predominant lactic acid bacteria in dolo and pito wort for the production of sorghum beer. J Appl Microbiol. 2007;103:765–777.

Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, Vuyst LD, Vandamme P, Huys G. Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. Appl Environ Microbiol. 2007;73(19):6262–9.

Scheirlinck I, Van der Meulen R, Schoor AV, Vancanneyt M, Vuyst LD, Vandamme P, Huys G. Taxonomic structure and stability of the bacterial community in Belgian sourdough ecosystems as assessed by culture and population fingerprinting. Appl Environ Microbiol. 2008;74(8):2414–23.

Scheirlinck I, Van der Meulen R, De Vuyst L, Vandamme P, Huys G. Molecular source tracking of predominant lactic acid bacteria in traditional Belgian sourdoughs and their production environments. J Appl Microbiol. 2009;106:1081–92.

Schleifer KH, Kilpper-BL lr R. Molecular and chemotaxonomic approaches to the classification of ctreplococci, enterococci and lactococci: A review. Sys Appl Microbial. 1987;10:1–10.

Schleifer KH, Kraus J, Dvorak C, Kilpper-bälz R, Collins MD, Fischer W. Transfer of *Streptococcus lactis* and related streptococci in the genus *Lactococcus* gen. nov. Int J Syst Appl Microbiol. 1985;6:183–195.

Schleifer KH, Ludwig W. Phylogenetic relationship of lactic acid bacteria. In wood BJB, Holzapfel WH, editors. The genera of lactic acid bacteria. London: Blackie Academic &Professional; 1995a. P. 7–18.

Schleifer KH, Ludwig W. Phylogeny of the genus *Lactobacillus* and related genera. Syst Appl Microbiol. 1995b;18:461–467.

Schleifer KH, Kilpper-BHlz R. Transfer of *Streptococcuss faecalis* and *Streptococcuss faecium* to the genus Entercoccus nom. rev. As *Enterococcus faecalis* comb. nov. Int J Syst Bacteriol. 1984;34:31–4.

Sedláček I, Holochová P, Mašlaňová I, Kosina M, Spröer C, Bryndová H, Vandamme P, Rudolf I, Hubálek Z, Švec P . *Enterococcus ureilyticus* sp. nov. and *Enterococcus rotai* sp. nov., two urease-producing enterococci from the environment. Int J Syst Evol Microbio. 2013;63:502–510.

Seegers JF. Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol. 2002;20(12):508–15.

Sengun IY, Nielsen DS, Karapinar M, Jakobsen M. Identification of lactic acid bacteria isolated from Tarhana, a traditional Turkish fermented food. Int J Food Microbiol. 2009;135:105–11.

Serhan M, Cailliez-Grimal C, Borges F, Revol-Junelles AM, Hosri C, Fanni J. Bacterial diversity of Dairyfeh, a Lebanese artisanal raw goat's milk cheese. Food Microbiol. 2009;26:645–52.

Sharpe ME, Garvie EI, Tilbury RH. Some slime-forming heterofermentative species of the genus Lactobacillus. Appl Micro biol. 1972;23:389–397.

Shaw BC, Harding CD. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J Appl Bacterial. 1984;56:25–40.

Shaw BG, Harding CD. A typical lactobacilli from vacuum-packaged meats: comparison by DNA hybridization cell composition and biochemical tests with a description of *Lactobacillus carnis* sp. nov. Syst App Microbial. 1985;6:291–297.

Shaw BG, Harding CD. *Leuconostoc gelidum* sp. nov. and *Leuconostoc carnosum* sp. nov. from chill-stored meats. Int J Syst Bacteriol. 1989;39:217–23.

Shuangquan B, Miyamoto T. Microflora in traditional fermented camel's milk from Inner Mongolia. China Milchwissenschaft. 2004;59:649–652.

Simova E, Beshkova D, Angelov A, Hristozova Ts, Frengova G, Spasov Z. Lactic acid bacteria and yeasts in kefir grains and kefir made from them. Int J Microbiol Biotechnol. 2002;28(1):1–6.

Simpson PJ, Ross RP, Fitzgerald GF, Stanton C. *Bifidobacterium psychraerophilum* sp. nov. and *Aeriscardovia aeriphila* gen. nov., sp. nov., isolated from a porcine caecum. Int J Syst Evol Microbiol. 2004;54:401–406.

Sistek V, Maheux AF, Boissinot M, Bernard KA, Cantin P, Cleenwerck I, De vos P, Bergeron MG. *Enterococcus ureasiticus* sp. nov. and *Enterococcus quebecensis* sp. nov., isolated from water. Int J Syst Evol Microbiol. 2012;62:1314–1320.

Smit G, Smit BA, Engels JM. Flavour formation by lacticacid bacteria and biochemical flavour profiling of chese products. FEMS Microbiol Rev. 2005;29:591–610.

Spicher G. Weitere Unterschungen über die Zusammensetzung und die Variabilitä t der Mikroflora handelsü blicher Sauerteig-Starter. Zeitschrift für Lebensmittel Untersuchung und Forschung. 1984;178:106–9.

Spicher G. Die Mikroflora des Sauerteiges. XXII. Mitteilung: Die in Weizensauerteigen vorkommenden Lactobacillen. Zeitschrift für Lebensmittel Untersuchung und. Forschung. 1987;184:300–3.

Spicher G. Die Mikroflora des Sauerteiges. I. Mitteilung: Untersuchungenüber die Art der in Sauerteigen anzutreffenden stäbchenförmigen Milchsaürebakterien (Genus Lactobacillus Beijerinck). Zeitblatt fur Bakteriologie II Abt. 1959;113:80–106.

Spicher G, Schröder R, Schöllhammer K. Die Mikroflora des Sauerteiges. VII. Mitteilung: Untersuchungen über die Art der in 'Reinzuchtsauern' auftretenden Hefen. Zeitschrift für Lebensmittel Untersuchung und. Forschung. 1979;169:77–81.

Stackebrandt E, Teuber M. Molecular taxonomy and phylogenetic position of lactic acid bacteria. Biochimie. 1988;70(317):324.

Stephan H, Neumann H. Technik der Roggen-Sauerteigfährung. In: Spicher G, Stephan H, editors. Handbuch Sauerteig: Biologie, Biochemie, Technologie. 5th ed. Hamburg: Behr's Verlag; 1999a. p. 161–245.

Stephan H, Neumann H. Technik der Weizenvorteigund Weizensauerteigführung. In: Spicher G, Stephan H, editors. Handbuch Sauerteig: Biologie, Biochemie, Technologie. 5th ed. Hamburg: Behr's Verlag; 1999b. p. 247–75.

Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol. 1997;36:1–29.

Sukontasing S, Tanasupawat S, Moonmangmee S, Lee JS, Suzuki K. *Enterococcus camelliae* sp. nov., isolated from fermented tea leaves in Thailand. Int J Syst. Evol Microbiol. 2007;57:2151–2154.

Sulieman AM, Ilayan AA, El faki AE. Chemical and microbiological quality of Garris, Sudanese fermented camel's milk product. Int J food Sci Technol. 2006;41(3):321–328.

Sun ZH, Liu WJ, Gao W, Yang M, Zhang JC, Wu L, Wang JG, Menghe BG, Sun TS, Zhang HP. Identification and characterization of the dominant lactic acid bacteria from kurut: the naturally fermented yak milk in Qinghai, China. J Gen Appl Microbiol. 2010;56:1–10.

Švec P, Devriese LA, Sedláček I, Baele M, Vancanneyt M, Haesebrouck F, Swings J, Doškař J. *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., isolated from water. Int J Syst Evol Microbiol. 2001;51:1567–74.

Švec P, Vancanneyt M, Devriese LA, Naser SM, Snauwaert C, Lefebvre K, Hoste B, Swings J. *Enterococcus aquimarinus* sp. nov., isolated from sea water. Int J Syst Evol Microbiol. 2005a;55:2183–7.

Švec P, Vancanneyt M, Koort J, Naser SM, Hoste B, Vihavainen E, Vandamme P, Swings J, Björkroth J. *Enterococcus devriesei* sp. nov., associated with animal sources. Int J Syst Evol Microbiol. 2005b;55:2479–84.

Švec P, Vancanneyt M, Sedláček I, Naser SM, Snauwaert C, Lefebvre K, Hoste B, Swings J. *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. Int J Syst Evol Microbiol. 2006;56:577–581.

Tailliez P. Mini-revue: les bactéries lactiques, ces êtres vivants apparus il y a près de 3 milliards d'années. Lait. 2001;81:1–11.

Tamime AY. Fermented milks: a historical food with modern applications-a review. Eur J Clin Nutr. 2002;56(Suppl)4:1–15.

Tamime AY. Production of Kefir, Koumiss and Other related Products. In: Tamime AY, editor. Fermented Milk Blackwell Science Ltd, Oxford, UK, 2006;174–216.

Tanasupawat S, Sukontasing S, Lee JS. *Enterococcus thailandicus* sp. nov., isolated from fermented sausage ('mum') in Thailand. Int J Syst Evol Microbiol. 2008;58:1630–4.

Taormina PJ. Implications of salt and sodium reduction on microbial food safety. Crit Rev Food Sci Nutr. 2010;50:209–27.

Teixeira LM, Carvalho M da GS, Espinola MMB, Steigerwalt AG, Douglas MP, Brenner DJ, Facklam RR. *Enterococcus porcinus* sp. nov. and *Enterococcus ratti* sp. nov., associated with enteric disorders in animals. Int J Syst Evol Microbiol. 2001;51:1737–1743.

Teuber M. The Genus *Lactococcus*. In: Wood BJB, Holzapfel WH, editors. The genera of lactic acid bacteria. London: Blackie; 1995. P. 173–234.

Thiercelin M. Sur un diplocoquc saprofjtc deI'iintestin susceptible de devcnir pathoehnc C'.R. Sot. Rioi. (Paris). 1899;51:269–271.

Thornley MJ. Observations on the microflora of minced chicken meat irradiated with 4 MeV cathode rays. J Appl Bacteriol. 1957;20:286–98.

Thunell RK. Taxonomy of the leuconostocs. J Dairy Sci. 1995;78:2514–22.

Tohno M, Kitahara M, Irisawa T, Masuda T, Uegaki R, Ohkuma M, Tajima K. Description of *Lactobacillus iwatensis* sp. nov., isolated from orchardgrass (Dactylis glomerata L.) silage, and *Lactobacillus backii* sp. nov. J Syst Evol Microbiol. 2013;63:3854–3860.

Tohno M, Kitahara M, Uegaki R, Irisawa T, Ohkuma M, Tajima K. *Lactobacillus hokkaidonensis* sp. nov., isolated from subarctic timothy grass (Phleum pratense L.) silage. Int J Syst Evol Microbiol. 2013a;63:2526–31.

Tohno M, Kitahara M, Irisawa T, Masuda T, Uegaki R. Ohkuma Moriya, Tajima K. Description of *Lactobacillus iwatensis* sp. nov., isolated from orchardgrass (Dactylis glomerata L.) silage, and *Lactobacillus backii* sp. nov. Int J Syst Evol Microbiol. 2013b;63:3854–60.

Tohno M, Kitahara M, Irisawa T, Inoue H, Uegaki R, Ohkuma M, Tajima K. *Lactobacillus oryzae* sp. nov., isolated from fermented rice grain (Oryza sativa L. subsp. japonica). Int J Syst Evol Microbiol. 2013c;63:2957–62.

Tooner JS. Koumiss in Mongol culture: past and present. Milk and milk products from Medieval to Modern Times In: P Lysaght ,editor. Canongate Press, Edinburgh 1994; p. 130–39.

Tyrrell GJ, Turnbull L, Teixeira LM, Lefebvre J, Carvalho MGS, Facklam RR, Lovgren M. *Enterococcus gilvus* sp. nov. and *Enterococcus pallens* sp. nov. isolated from human clinical specimens. J Clin Microbiol. 2002;40:1140–5.

Tzanetakis N, Litopoulou-Tzanetaki E. Biochemical activities of *Pediococcus* pentosaceus isolates of dairy origin. J Dairy Sci. 1989;72(4):859–63.

Uchida K, Hirata M, Motoshima H, Urashima T, Arai I. Microbiota of'airag', 'tarag' and other kinds of fermented dairy products from nomad in Mongolia. Anim Sci J. 2007;78(6):650–8.

Urso R, Comi G, Luca C. Ecology of lactic acid bacteria in Italian fermented sausages: isolation, identification and molecular characterization. Syst Appl Microbiol. 2006;29:671–80.

Van den Berg G. Semi-hard cheeses. In: Tamime, A.Y., Law, B.A. editors, Mechanisation and Automation in Dairy Technology. Academic Press, Sheffield. 2000.

Van Hoorde K, Verstraete T, Vandamme P, Huys G. Diversity of lactic acid bacteria in two Flemish artisan raw milk Gouda-type cheeses. Food Microbiol. 2008;25:929–35.

Vedamuthu ER. The dairy Leuconostoc: use in dairy products. J Dairy Sci. 1994;77(9):2725–2737.

Venturi M, Guerrini S, Vincenzini M. Stable and non-competitive association of Saccharomyces cerevisiae, Candida milleri and Lactobacillus sanfranciscensis during manufacture of two traditional sourdough baked goods. Food Microbiol. 2012;31:107–15.

Vogel RF, Knorr R, Mü ller MRA, Steudel U, Gänzle, MG, Ehrmann MA. Non-dairy lactic acid fermentations: the cereal world. Antonie Van Leeuwenhoek. 1999;76:403–411.

Vuyst LD, Schrijvers V, Paramithiotis S, Hoste B, Vancanneyt M, Swings J, Kalantzopoulos G, Tsakalidou E, Messens W. The biodiversity of lactic acid bacteria in Greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. Appl Environ Microbiol. 2002;68(12):6059–69.

Wallbanks S, Martinez-Murcia AJ, Fryer JL, Phillips BA, Collins MD. 16S rRNA sequence determination for members of the genus Carnobacterium and related lactic acid bacteria and description of *Vagococcus salmoninurum* sp. nov. Int J Syst Evol Bacteriol. 1990;40:224–230.

Ward DM, Weller R, Bateson MM. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature. 1990;345:63–5.

Walstra P, Moomen, geurts TJ. Dutch-type varieties, in Cheese: chemistry. Physic and Microbiology, Vol. 2, In: P.F. Fox, editor, Chapman & Hall, London, 1993; p. 39–82.
Watanabe K, Fujimoto J, Sasamoto M, et al. Diversity of lactic acid bacteria and yeasts in Airag and Tarag, traditional fermented milk products of Mongolia. World J Microbiol Biotechnol. 2008;24:1313–25.
Williams REO, Hirch A, Cowan ST. *Aerococcus*, a new bacterial genus. J Gen Microbiol. 1953;8:475–80.
Williams AM, Farrow JAE, Collins MD. Validation list no. 31. Int. J. Syst. Bacteriol. 1989;39:495–97.
Williams AM, Fryers JL, Collins M. *Lactococcus piscium* sp. nov. a new Lactococcus species from salmonid fish. FEMS Microbiol Lett. 1990;68:109–14.
Collins MD, Rodrigues UM, Pigott NF, Facklam RR. *Enterococcus* species from human sources. Lett Appl Microbiol. I991;12:95–98.
Yang CH, Crowley DE, Borneman J, Keen NT. Microbial phyllosphere populations are more complex than previously realized. Proc Natl Acad Sci USA. 2001;98:3889–94.
Yao AK, Kadio G, Coulybaly A, Agbo GZ. Production du 'Tchapalo' a' partir du sorgho en Côte d'Ivoire. In: Menyonga JM, Bezuneh T, Nwasike CC, Sedogo PM, Tenkouano A editors Processing and industrial utilization of sorghum and related cereals in Africa. Proceeding of the OUA/STRCSAFGRAD regional symposium. Ouagadou-gou, Burkina Faso, 22–26 November 1993, 1995; pp 55–60.
Yi EJ, Yang JE, Lee JM, Park YJ, Park SY, ShinHS, Kook MC, Yi TH. *Lactobacillus yonginensis* sp. nov., a lactic acid bacterium with ginsenoside converting activity isolated from Kimchi. Int J Syst Evol Microbiol. 2013. doi:10.1099/ijs.0.045799-0.
Yoon JH, Kang SS, Mheen TI, Ahn JS, Lee HJ, Kim TK, Park CS, Kho YH, Kang KH, Park YH. *Lactobacillus kimchii* sp. nov., a new species from kimchi. Int J Syst Evol Microbiol. 2000;50:1789–95.
Yu J, Du XH, Wang WH, Zhang JC, Liu WJ, Sun ZH, Sun TS, Zhang HP. Phenotypic and genotypic characteristics of lactic acid bactereia isolated from sour congee in Inner Mongolia of China. J Gen Appl Microbiol. 2011;57:197–206.
Zamfir M, Vancanneyt M, Makras L, Vaningelgem F, efebvre KL, Pot B, Swings J, De Vuyst L. Biodiversity of lactic acid bacteria in Romanian dairy products. Syst Appl Microbiol. 2006;29:487–495.
Zapparoli G, De Benedictis P, Salardi C, Veneri G, Torriani S, Dellaglio F. Lactobacilli of sourdoughs from Verona bakery: a preliminary investigation. Adv Food Sci. 1996;18:163–6.
Zhang HP, Xu J, Wang JG, Menghebilige, Sun TS, Li HP, Guo MR. A survey on chemical and microbiological composition of Kurut, naturally fermented yak milk from Qinghai in China. Food Control. 2008;19:578–586.
Zhang WY, Zhang HP. Fermentation and koumiss. In: Hui YH, editor. Handbook of animal-based fermented foods and beverages. 2nd ed. Boca Raton: CRC Press; 2012. p. 165–172.
Zhang JC, Liu WJ, Sun ZH, Bao QH, Wang F, Yu J, Chen W, Zhang HP. Diversity of lactic acid bacteria and yeasts in traditional sourdoughs collected from western region in Inner Mongolia of China. Food Control. 2011;22:767–74.
Zhu G, Zhang Y. Current conditions and developing approaches of yak production in China. Chin J Anim Sci (in Chinese). 2005;41(1):61–3.
Zou YQ, Liu F, Fang CX, Wan DW, Yang RT, Su QQ, Yang RF, Zhao J. *Lactobacillus shenzhenensis* sp. nov., isolated from a fermented dairy beverage. Int J Syst Evol Microbiol. 2013;63:1817–1823.

Chapter 3
Genomics of Lactic Acid Bacteria

Wenyi Zhang and Heping Zhang

Abstract Lactic acid bacteria (LAB) are 'generally recognised as safe' microorganisms, and some of them were given the 'Qualified Presumption of Safety' status by the European Food Safety Authority. Due to their significant contribution to various industrial applications, many of these organisms were subjected to full genome sequencing projects. Together with the increased amount of published transcriptomics and proteomics data across the entire genome, we have unprecedented opportunities to revisit the important traits of LAB. More importantly, this increased amount of data will aid our understanding of the mechanisms underlying the interaction between LAB and human beings. In this chapter, we focus on the current research progress on LAB genomics and pay particular attention to those species that have played major roles in lactic fermentations. The key features of the genomes and the mechanisms that have been correlated to the beneficial actions of LAB are also discussed. We aim to provide some basic information to our colleagues within the scientific community.

Keywords Lactic acid bacteria · Genomics · Key features

3.1 Introduction

Lactic acid bacteria (LAB) are generally accepted as a group of Gram-positive, low-GC-content, nonmotile, non-spore-forming, rod-shaped and coccus-shaped microorganisms that can ferment hexose carbohydrates under microaerophilic to

W. Zhang (✉) · H. Zhang
Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education of China, Inner Mongolia Agricultural University, Hohhot, China
e-mail: zhangwenyizi@163.com

H. Zhang
e-mail: hepingdd@vip.sina.com

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_3,
© Springer Science+Business Media Dordrecht 2014

strictly anaerobic conditions to produce mainly lactic acid. Pioneering scientific and technical developments of LAB were undertaken during the latter part of the nineteenth century. Thereafter, at the beginning of the 1900s, the concept of LAB was developed. As documented in the long history of the use of LAB, these organisms were first discovered by Pasteur in 1857, and the isolation of a pure culture was then achieved by Lister in 1873. The archaeological literature, however, shows that the use of LAB may extend to 8,000 years ago (Curry 2013).

The early definition of LAB was mainly based on their morphological and physiological features. It is not surprising that the initial classification of LAB was restricted to *Betabacterium*, *Thermobacterium*, *Streptobacterium*, *Streptococcus*, *Betacoccus*, *Microbacterium* and *Tetracoccus* (Stiles and Holzapfel 1997). Currently, the incorporation of multiple modern methods, such as the analysis of the cell wall composition, the total soluble cytoplasmatic proteins, and the electrophoretic mobility of certain enzymes, provides sufficient justification for this grouping (Klein et al. 1998). Molecular typing methods have also been used to distinguish bacteria at the intraspecific level (Pal et al. 2012; Ben Amor et al. 2007; Rossetti and Giraffa 2005). According to taxonomy, the LAB were expanded to *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Oenococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Carnobacterium* and *Tetragenococcus* (Klein et al. 1998), and these genera were subdivided into a considerable number of species that can be widely detected in various natural environments, e.g., dairy products, meats, vegetables, blood, swine, small intestine and colon. *Lactobacillus*, which includes 147 recognised species, the largest and most diverse genus documented to date (Claesson et al. 2008).

Traditionally, LAB are often used in the fermentation of dairy products, sourdough, alcohol and vegetables (Stiles 1996). In addition to the preservation roles displayed by the production of lactic acid, their contribution to flavour compound, texture and nutritive value is also desirable (Tafti et al. 2013; Martinez-Cuesta Mdel et al. 2013). The particular interest in LAB may date back to the turn of the twentieth century, when Elie Metchnikoff, a Russian immunologist at the Pasteur Institute in Paris, suggested that the consumption of Lactobacilli is good for people's health and thus longevity. At that time, not all fellow scientists agreed with his view due to the limited clinical evidence. Although today's researchers continue to explore the new health-promoting effects of certain LAB strains (Shiby and Mishra 2013; Aureli et al. 2011), the successful application of these organisms to decrease the recurrence of local infections (Falagas et al. 2008), stimulate the immuno regulatory response (Foligne et al. 2007; Ya et al. 2008), control cholesterol levels (Gilliland 1990) and inhibit tumour growth (Gorbach 1990), have been clearly demonstrated. More recently, a LAB strain that exerts both preventive and ameliorative effects on oral glucose tolerance in rats was discovered (Zhang et al. 2013).

3.1.1 Lactic Acid Bacteria and Genome Sequencing Projects

LAB are 'generally recognised as safe' (GRAS) microorganisms (Adams 1999), and some of them were given the 'Qualified Presumption of Safety' (QPS) status by the European Food Safety Authority (EFSA) (Taverniti and Guglielmetti 2012). Due to their significant contribution to various industrial applications, many of these organisms were subjected to full genome sequencing projects. Together with the increased amount of published transcriptomics and proteomics data across the entire genome, we have unprecedented opportunities to revisit the important traits of LAB. More importantly, this increased amount of data will aid our understanding of the mechanisms underlying the interaction between LAB and human beings.

In 2001, the first genome of an LAB, namely the *Lactococcus lactis* IL1403 strain, which is commonly used as a starter for cheese making, was sequenced and published (Bolotin et al. 1999). The availability of such a good model bacterium was recognised as a boost to speed-up the achievements in re-routing the metabolic fluxes of this LAB (Vadeboncoeur and Moineau 2004). Since then, it became possible to increase the production of diacetyl and vitamins and to transform this bacterium from a homolactic fermenter to a homoalanine fermenter (Vadeboncoeur and Moineau 2004; Burgess et al. 2004). Striking advances in genome sequencing were achieved at the 8th Symposium on LAB held in August 2005 in Egmond aan Zee, the Netherlands. At that time, many of the available genome sequences were contributed by the Department of Energy-Joint Genome Institute (JGI) in collaboration with the Lactic Acid Bacteria Genome Consortium (LABGC), which is composed of 10 US scientists representing seven universities in the US. LAB species representing considerable diversity in ecological habitat and roles were studied (Klaenhammer et al. 2002). At present, more than 75 LAB genomes have been completed (Table 3.1), and approximately 80 genome sequencing projects are still in progress throughout the world. An analysis of the finished genomes reveals that a majority of the strains belong to the genus *Lactobacillus*, i.e. 15 different species. A high coverage was also found in species of the genus *Leuconostoc* likely due to their importance in vegetable fermentations in Korea. In some cases, a single strain derived from the same original isolate was sequenced more than once by different bacterial collection centres or research institutes. For instance, *Lactobacillus reuteri* JCM 1112^{T} and *Lactobacillus reuteri* DSM 20016^{T}, which originated from the isolate *L. reuteri* F275, were both sequenced. It was expected that there would be minimum differences between the two genomes; however, even different genome sizes were found, which provides information on the stability of the genome during regular lab culturing and transfers. First, these data indicate the tendency of LAB strains to undergo genome changes under certain lab conditions, which highlights the importance of a stability assessment of the strain of interest before any practical use. On the other hand, to what extent the strain can function as its original isolate is very important to researchers. The loss of phenotypes that are relevant to its typical properties can be somewhat misleading during normal tests.

Table 3.1 General information of completed LAB genomes[a]

Strain	ID	Plasmids	Size (Mb)	GC%	Gene	Protein	References
Lactobacillus plantarum WCFS1	NC_004567.2	3	3.35	44.4	3,175	3,064	Kleerebezem et al. (2003)
Lactobacillus plantarum 16	NC_021514.1	10	3.36	44.3	3,171	3,088	Crowley et al. (2013)
Lactobacillus plantarum ZJ316	NC_020229.1	3	3.3	44.4	3,352	3,276	Li et al. (2013)
Lactobacillus plantarum JDM1	NC_012984.1	0	3.2	44.7	3,028	2,947	Zhang et al. (2011)
Lactobacillus plantarum ST-III	NC_014554.1	1	3.31	44.5	3,182	3,038	Wang et al. (2010)
Lactobacillus plantarum P-8	NC_021224.1	6	3.23	44.6	3,206	3,090	–
Lactobacillus acidophilus NCFM	NC_006814.3	0	2	34.7	1,938	1,864	Altermann et al. (2005)
Lactobacillus acidophilus La-14	NC_021181.2	0	1.99	34.7	1,957	1,876	Stahl and Barrangou (2013)
Lactobacillus acidophilus 30SC	NC_015214.1	2	2.1	38.1	2,134	2,059	Oh et al. (2011)
Lactobacillus brevis ATCC367	NC_008497.1	2	2.35	46.1	2,351	2,218	Makarova et al. (2006)
Lactobacillus brevis KB290	NC_020819.1	9	2.59	45.6	2,582	2,582	Fukao et al. (2013)
Lactobacillus johnsonii NCC533	NC_005362.1	0	1.99	34.6	1,918	1,821	Pridmore et al. (2004)
Lactobacillus johnsonii DPC6026	NC_017477.1	0	1.97	34.8	1,854	1,772	Guinane et al. (2011)
Lactobacillus johnsonii FI9785	NC_013504.1	2	1.79	34.4	1,807	1,735	Wegmann et al. (2009)
Lactobacillus casei ATCC334	NC_008526.1	1	2.93	46.6	2,922	2,764	Cai et al. (2009)
Lactobacillus casei BL23	NC_010999.1	0	3.08	46.3	3,072	2,997	Maze et al. (2010)
Lactobacillus casei Zhang	NC_014334.1	1	2.9	46.4	2,949	2,847	Zhang et al. (2010)
Lactobacillus casei LC2W	NC_017473.1	1	3.08	46.4	3,264	3,164	Chen et al. (2011)
Lactobacillus casei BD-II	NC_017474.1	1	3.13	46.3	3,300	3,199	Ai et al. (2011)
Lactobacillus casei LOCK919	NC_021721.1	1	3.14	46.2	3,178	3,103	–
Lactobacillus casei W56	NC_018641.1	1	3.13	46.3	3,234	3,126	Hochwind et al. (2012)
Lactobacillus rhamnosus Lc705	NC_013199.1	1	3.03	46.6	3,033	2,957	Kankainen et al. (2009)
Lactobacillus rhamnosus GG	NC_017482.1	0	3.01	46.7	2,905	2,834	Kankainen et al. (2009)
Lactobacillus rhamnosus ATCC 8530	NC_017491.1	0	2.96	46.8	2,977	2,886	Pittet et al. (2012)
Lactobacillus rhamnosus LOCK900	NC_021723.1	1	2.88	46.8	2,901	2,827	–
Lactobacillus rhamnosus LOCK908	NC_021725.1	1	2.99	46.8	2,999	2,924	–

(continued)

Table 3.1 (continued)

Strain	ID	Plasmids	Size (Mb)	GC%	Gene	Protein	References
Lactobacillus helveticus H10	NC_017467.1	1	2.17	36.8	2,148	1,978	Zhao et al. (2011)
Lactobacillus helveticus R0052	NC_018528.1	0	2.13	36.8	2,084	2,011	Tompkins et al. (2012)
Lactobacillus helveticus CNRZ32	NC_021744.1	0	2.23	38.9	1,923	1,685	–
Lactobacillus helveticus DPC 4571	NC_010080.1	0	2.08	37.1	1,838	1,610	Callanan et al. (2008)
Lactobacillus sanfranciscensis TMW 1.1304	NC_015978.1	2	1.38	34.8	1,519	1,284	Vogel et al. (2011)
Lactobacillus ruminis ATCC27782	NC_015975.1	0	2.07	43.5	2,054	1,862	–
Lactobacillus sakei 23 K	NC_007576.1	0	1.88	41.3	1,955	1,871	Chaillou et al. (2005)
Lactobacillus gasseri ATCC33323	NC_008530.1	0	1.9	35.3	1,898	1,755	Azcarate-Peril et al. (2008)
Lactobacillus salivarius ssp. *salivarius* UCC118	NC_007929.1	3	2.13	33	2,182	2,013	Claesson et al. (2006)
Lactobacillus salivarius ssp. *salivarius* CECT 5713	NC_017481.1	3	2.14	33	–	–	Kergourlay et al. (2012)
Lactobacillus delbrueckii ssp. *bulgaricus* ATCC11842	NC_008054.1	0	1.86	49.7	2,184	1,529	–
Lactobacillus delbrueckii ssp. *bulgaricus* ATCC BAA-365	NC_008529.1	0	1.9	49.7	2,026	1,708	–
Lactobacillus delbrueckii ssp. *bulgaricus* ND02	NC_014727.1	1	2.13	49.6	2,183	2,018	Sun et al. (2011)
Lactobacillus delbrueckii ssp. *bulgaricus* 2038	NC_017469.1	0	1.87	49.7	1,792	1,792	Hao et al. (2011)
Lactobacillus reuteri SD2112	NC_015697.1	0	2.32	39	2,425	2,300	–
Lactobacillus reuteri DSM20016	NC_009513.1	4	2	38.9	2,027	1,900	–
Lactobacillus reuteri 15007	NC_021494.1	0	2.09	38.9	–	–	–
Lactobacillus reuteri JCM1112	NC_010609.1	6	2.04	38.9	1,901	1,820	–
Lactobacillus reuteri TD1	NC_021872.1	0	2.15	38.8	2,061	1,945	–
Lactobacillus fermentum IFO 3956	NC_010610.1	0	2.1	51.5	1,912	1,843	Morita et al. (2008)
Lactobacillus fermentum F-6	NC_021235.1	0	2.06	51.7	2,090	2,006	–
Lactobacillus fermentum CECT 5716	NC_017465.1	0	2.1	51.5	1,149	1,051	Jimenez et al. (2010)
Lactococcus lactis ssp. *cremoris* SK11	NC_008527.1	5	2.56	35.8	2,739	2,501	–
Lactococcus lactis ssp. *cremoris* MG1363	NC_009004.1	0	2.5	35.7	2,597	2,434	Wegmann et al. (2007)
Lactococcus lactis ssp. *cremoris* UC509.9	NC_019435.1	8	2.46	35.8	2,401	2,109	Ainsworth et al. (2013)
Lactococcus lactis ssp. *cremoris* NZ9000	NC_017949.1	0	2.53	35.7	2,594	2,510	Linares et al. (2010)

(continued)

Table 3.1 (continued)

Strain	ID	Plasmids	Size (Mb)	GC%	Gene	Protein	References
Lactococcus lactis ssp. *cremoris* A76	NC_017492.1	4	2.58	35.9	2,845	2,769	–
Lactococcus lactis ssp. *lactis* IO-1	NC_020450.1	0	2.42	35.1	2,318	2,224	Kato et al. (2012)
Lactococcus lactis ssp. *lactis* CV56	NC_017486.1	5	2.52	35.1	2,549	2,408	Gao et al. (2011)
Lactococcus lactis ssp. *lactis* IL1403	NC_002662.1	0	2.37	35.3	2,425	2,321	Bolotin et al. (1999)
Lactococcus lactis ssp. *lactis* KF147	NC_013656.1	1	2.64	34.9	2,662	2,473	Siezen et al. (2010)
Leuconostoc carnosum JB16	NC_018673.1	4	1.77	37.1	1,769	1,691	Jung et al. (2012a)
Leuconostoc gelidum JB7	NC_018631.1	0	1.89	36.7	1,875	1,796	Jung et al. (2012b)
Leuconostoc mesenteroides J18	NC_016805.1	5	2.02	37.7	2,020	1,937	Jung et al. (2012c)
Leuconostoc mesenteroides ATCC8293	NC_008531.1	1	2.04	37.7	2,106	2,003	–
Leuconostoc citreum KM20	NC_010471.1	4	1.9	38.9	1,903	1,820	Kim et al. (2008)
Leuconostoc kimchii IMSNU 11154	NC_014136.1	5	2.1	37.9	2,209	2,129	Oh et al. (2010)
Leuconostoc gasicomitatum LMG 18811	NC_014319.1	0	1.95	36.7	1,993	1,912	Johansson et al. (2011)
Oenococcus oeni PSU-1	NC_008528.1	0	1.8	37.9	1,864	1,691	Makarova et al. (2006)
Streptococcus thermophilus LMD-9	NC_008532.1	2	1.91	39.1	2,004	1,711	–
Streptococcus thermophilus JIM8232	NC_017581.1	0	1.93	38.9	2,230	2,145	Delorme et al. (2011)
Streptococcus thermophilus ND03	NC_017563.1	0	1.83	39	2,038	1,919	Sun et al. (2010)
Streptococcus thermophilus MN-ZLW-002	NC_017927.1	0	1.85	39.1	2,046	1,910	Kang et al. (2012)
Streptococcus thermophilus CNRZ1066	NC_006449.1	0	1.8	39.1	1,999	1,914	Bolotin et al. (2004)
Streptococcus thermophilus LMG18311	NC_006448.1	0	1.8	39.1	1,973	1,888	Pastink et al. (2009)
Pediococcus pentosaceus ATCC25745	NC_008525.1	0	1.8	37.4	1,844	1,752	Diep et al. (2006)
Weissella koreensis KACC 15510	NC_015759.1	1	1.73	35.5	1,750	1,672	Lee et al. (2011)

[a] Genome information is extracted from NCBI website (http://www.ncbi.nlm.nih.gov/genome/genomes/)

With the exception on the general genome information summarised above, an increasing amount of draft genomes are being submitted to the public database (Table 3.2). For some specific purpose, this strategy is cost-effective compared with the costs associated with a full-genome sequencing project. Interestingly, multiple strains from several species, such as *L. casei*, *L. crispatus*, *L. iners*, *L. delbrueckii*, *L. rhamnosus, Lactoc. lactis* ssp. *cremoris*, *Oenococcus oeni*, *Streptococcus thermophilus* and *Leuconostoc citreum*, have been submitted, suggesting an explosion on the investigation of interspecies biodiversity. A research group from China, namely the Key Laboratory of Dairy Biotechnology and Engineering Ministry of Education at Inner Mongolia Agricultural University, is known to have made many contributions. As part of their genome sequencing programs, type strains of the genus *Lactobacillus* were sequenced and will be placed in a public domain for comparative genome analysis. Within the current scientific community, 'Pet' *Lactobacillus* strains have always been selected and subjected to sequencing; thus, a bias towards the published genomes can be observed. In particular, most of the sequenced strains are well-studied probiotics, as was observed with the first release of the genome of *L. plantarum* WCSF1, a species belonging to the genus *Lactobacillus*, in 2003. This clearly limits our knowledge of the biodiversity and evolution of *Lactobacillus*. Due to the lack of key taxonomic species within *Lactobacillus*, the phylogeny relationships between these species remain somewhat obscure, although many studies have attempted to address these issues. The study of these species appears as 'A good rain', which reveals that the genetic diversity of *Lactobacillus* is higher than the majority of traditionally well-defined families but lower than an order, providing a comprehensive basis for nearly all aspects of the current research on LAB biology (Prof. Zhang, personal communication). Accompanying the improved sequencing technologies and bioinformatic tools, multiple strains that occupy a range of environmental niches have become available (Nelson 2003). It is likely that we are now able to describe the heterogeneous LAB species from a pan-genome level (Hao et al. 2011), but it should be noted that full genome sequences with complete information on gene order and collinearity are still needed for a detailed comparative genomic analysis.

In this chapter, we focus on the current research progress on LAB genomics and pay particular attention to those species that have played major roles in lactic fermentations. The key features of the genomes and the mechanisms that have been correlated to the beneficial actions of LAB are also discussed. We aim to provide some basic information to our colleagues within the scientific community.

3.2 General Genome Features

The genomes of LAB consist of single circular chromosomes with a length ranging from 1.3 to 3.35 Mbp; although some of these species harbour plasmids, all contain IS elements and numerous pseudogenes (Vogel et al. 2011; Goh and Klaenhammer 2009; Zhu et al. 2009). The first genome from *L. sanfranciscensis*, a

Table 3.2 General information of draft LAB genomes[a]

Strain	Size (Mb)	GC%	Gene	Protein
Lactobacillus acidipiscis KCTC 13900	2.29	39.1	–	–
Lactobacillus acidophilus ATCC 4796	2.02	34.6	2,084	2,020
Lactobacillus amylolyticus DSM 11664	1.54	38.2	1,746	1,684
Lactobacillus animalis KCTC 3501	1.88	41.1	–	–
Lactobacillus antri DSM 16041	2.3	51.1	2,286	2,224
Lactobacillus brevis gravesensis ATCC 27305	3.14	40	3,106	3,041
Lactobacillus buchneri ATCC 11577	2.91	39.5	3,065	3,002
Lactobacillus casei 12A	2.89	46.4	2,808	2,702
Lactobacillus casei 21 1	3.22	46.2	3,241	3,080
Lactobacillus casei 32G	3.01	46.4	3,094	2,920
Lactobacillus casei A2 362	3.36	46.1	3,398	3,262
Lactobacillus casei CRF28	3.04	46.3	3,044	2,911
Lactobacillus casei Lc 10	2.95	46.4	2,888	2,779
Lactobacillus casei Lpc 37	3.08	46.2	2,967	2,860
Lactobacillus casei M36	3.15	46.3	3,126	3,001
Lactobacillus casei T71499	3	46.2	2,915	2,795
Lactobacillus casei UCD174	3.07	46.4	3,153	3,018
Lactobacillus casei UW1	2.87	46.4	2,964	2,826
Lactobacillus casei UW4	2.76	46.4	2,808	2,689
Lactobacillus casei UW4	2.63	46.4	–	–
Lactobacillus catenaformis OT 569	1.92	32.1	–	–
Lactobacillus coleohominis 101 4 CHN	1.73	41.3	1,709	1,652
Lactobacillus coryniformis CECT 5711	–	–	–	–
Lactobacillus coryniformis KCTC 3167	–	–	–	–
Lactobacillus coryniformis torquens KCTC 3535	–	–	–	–
Lactobacillus crispatus 125 2 CHN	2.31	36.7	2,139	2,082
Lactobacillus crispatus 214 1	2.07	36.8	2,221	2,163
Lactobacillus crispatus CTV 05	2.36	37.1	2,431	2,248
Lactobacillus crispatus FB049 03	2.46	36.9	2,509	2,433
Lactobacillus crispatus FB077 07	2.7	36.7	2,716	2,643
Lactobacillus crispatus JV V01	2.22	36.9	2,278	2,209
Lactobacillus crispatus MV 1A US	2.25	36.8	2,483	2,339
Lactobacillus crispatus MV 3A US	2.44	36.7	2,387	2,330
Lactobacillus curvatus CRL 705	1.84	41.8	1,918	1,862
Lactobacillus delbrueckii bulgaricus CNCM I 1519	–	–	–	–
Lactobacillus delbrueckii bulgaricus CNCM I 1632	–	–	–	–
Lactobacillus delbrueckii bulgaricus PB2003 044T34	1.98	49.9	1,981	1,909
Lactobacillus delbrueckii lactis DSM 20072	2.07	49.8	2,084	2,006
Lactobacillus equicursoris CIP 110162	2.15	47.8	1,938	1,935
Lactobacillus farciminis KCTC 3681	2.5	36.4	–	–
Lactobacillus fermentum 28 3 CHN	2.03	52.1	1,933	1,880
Lactobacillus fermentum ATCC 14931	1.87	52.8	1,929	1,866
Lactobacillus florum 2F	1.26	41.6	1,217	1,190

(continued)

Table 3.2 (continued)

Strain	Size (Mb)	GC%	Gene	Protein
Lactobacillus fructivorans KCTC 3543	1.37	38.9	–	–
Lactobacillus gasseri 202 4	1.82	34.9	1,821	1,773
Lactobacillus gasseri 224 1	2.01	35	2,354	2,252
Lactobacillus gasseri CECT 5714	1.91	35	1,843	1,660
Lactobacillus gasseri JV V03	2.01	34.6	2,036	1,977
Lactobacillus gasseri MV 22	1.93	35	2,028	1,626
Lactobacillus gastricus PS3	1.9	41.8	1,404	1,269
Lactobacillus gigeriorum CRBIP 24 85	1.93	36.9	1,895	1,892
Lactobacillus helveticus DSM 20075	2.02	36.8	2,129	2,078
Lactobacillus helveticus MTCC 5463	2.05	36.7	2,307	2,239
Lactobacillus hilgardii ATCC 8290	2.72	39.6	2,854	2,791
Lactobacillus hominis CRBIP 24 179	1.93	35.1	1,895	1,891
Lactobacillus iners AB 1	1.29	32.7	1,258	1,209
Lactobacillus iners ATCC 55195	1.24	32.5	1,197	1,144
Lactobacillus iners DSM 13335	1.28	32.5	1,264	1,214
Lactobacillus iners LEAF 2052A d	1.32	32.7	1,311	1,256
Lactobacillus iners LEAF 2053A b	1.37	32.3	1,333	1,277
Lactobacillus iners LEAF 2062A h1	1.3	32.6	1,318	1,265
Lactobacillus iners LEAF 3008A a	1.27	32.4	1,265	1,210
Lactobacillus iners LactinV 01V1 a	1.29	32.6	1,581	1,527
Lactobacillus iners LactinV 03V1 b	1.3	32.7	1,513	1,459
Lactobacillus iners LactinV 09V1 c	1.31	32.6	1,415	1,361
Lactobacillus iners LactinV 11V1 d	1.31	32.6	1,393	1,338
Lactobacillus iners SPIN 1401G	1.28	32.5	1,270	1,238
Lactobacillus iners SPIN 2503V10 D	1.28	32.6	1,326	1,273
Lactobacillus iners UPII 143 D	1.26	32.5	1,243	1,186
Lactobacillus iners UPII 60 B	1.32	32.7	1,330	1,276
Lactobacillus ingluviei Autruche 4	2.01	50.9	–	–
Lactobacillus jensenii 1153	1.75	34.5	1,704	1,347
Lactobacillus jensenii 115 3 CHN	1.65	34.1	1,520	1,470
Lactobacillus jensenii 269 3	1.69	34.4	1,624	1,575
Lactobacillus jensenii 27 2 CHN	1.64	34.1	1,590	1,506
Lactobacillus jensenii JV V16	1.6	34.4	1,518	1,450
Lactobacillus jensenii SJ 7A US	1.72	34.2	1,679	1,630
Lactobacillus johnsonii ATCC 33200	1.78	34.5	1,896	1,838
Lactobacillus johnsonii pf01	1.88	34.5	1,889	1,846
Lactobacillus kisonensis F0435	2.99	41.6	3,373	3,325
Lactobacillus malefermentans KCTC 3548	2	41	–	–
Lactobacillus mali KCTC 3596	2.15	36.2	2,169	2,139
Lactobacillus mucosae LM1	2.21	45.9	2,097	2,039
Lactobacillus oris F0423	2.17	49.7	2,138	2,050
Lactobacillus oris PB013 T2 3	2.12	49.8	2,105	2,038
Lactobacillus paracasei 8700 2	3.03	46.3	2,890	2,781
Lactobacillus paracasei ATCC 25302	2.99	46.5	3,103	3,042

(continued)

Table 3.2 (continued)

Strain	Size (Mb)	GC%	Gene	Protein
Lactobacillus parafarraginis F0439	2.85	45.6	3,229	3,183
Lactobacillus pasteurii CRBIP 24 76	1.91	38.6	1,856	1,845
Lactobacillus pentosus KCA1	3.43	46.4	3,061	2,967
Lactobacillus plantarum ATCC 14917	3.21	44.5	3,223	3,154
Lactobacillus plantarum NC8	3.21	44.6	3,007	2,868
Lactobacillus plantarum UCMA 3037	3.11	44.5	2,997	2,932
Lactobacillus pobuzihii E100301	2.35	37.7	2,235	2,154
Lactobacillus reuteri 100 23	2.31	38.7	2,269	2,181
Lactobacillus reuteri ATCC 53608	2.02	37.6	1,910	1,864
Lactobacillus reuteri CF48 3A	2.11	38. 7	2,223	2,164
Lactobacillus reuteri MM2 3	2.02	38.7	2,105	2,045
Lactobacillus reuteri MM4 1A	2.07	38.8	2,226	2,095
Lactobacillus rhamnosus ATCC 21052	2.88	46.7	3,063	3,014
Lactobacillus rhamnosus HN001	2.91	46.6	2,864	2,811
Lactobacillus rhamnosus LMS2 1	3.16	46.5	3,209	3,155
Lactobacillus rhamnosus LRHMDP2	2.91	46.5	2,965	2,908
Lactobacillus rhamnosus LRHMDP3	2.91	46.6	2,983	2,921
Lactobacillus rhamnosus MTCC 5462	2.52	46.9	3,308	3,255
Lactobacillus rhamnosus R0011	2.9	46.7	2,782	2,719
Lactobacillus rossiae DSM 15814	2.96	43.6	–	–
Lactobacillus ruminis ATCC 25644	2.11	43.7	2,313	2,251
Lactobacillus ruminis ATCC 25644	2.07	43.7	2,205	2,153
Lactobacillus ruminis SPM0211	2.17	43.7	2,391	2,326
Lactobacillus saerimneri 30a	1.63	42.5	1,583	1,519
Lactobacillus salivarius ACS116VCol5a	2.04	32.7	2,184	2,121
Lactobacillus salivarius ATCC11741	2.02	32.5	2,046	1,976
Lactobacillus salivarius GJ24	2	33	1,962	1,876
Lactobacillus salivarius NIAS840	2.05	33	1,996	1,869
Lactobacillus salivarius SMXD51	1.97	32.9	1,873	1,771
Lactobacillus suebicus KCTC3549	2.66	39	–	
Lactobacillus ultunensis DSM16047	2.25	36	2,271	2,210
Lactobacillus vaginalis ATCC49540	1.88	40.6	1,299	1,257
Lactobacillus versmoldensis KCTC3814	2.4	38.3	–	–
Lactobacillus vini DSM 20605	2.2	37.6	2,232	2,174
Lactobacillus vini JP789	2.28	37.7	–	–
Lactococcus lactis ssp. *cremoris* CNCM I-1631	2.51	34.9	2,630	2,579
Lactococcus lactis ssp. *cremoris* TIFN1	2.68	35.5	2,976	2,754
Lactococcus lactis ssp. *cremoris* TIFN3	2.73	35.5	3,112	2,891
Lactococcus lactis ssp. *cremoris* TIFN5	2.54	35.5	2,601	2,232
Lactococcus lactis ssp. *cremoris* TIFN6	2.59	35.7	2,728	2,334
Lactococcus lactis ssp. *cremoris* TIFN1	2.63	35.3	2,870	2,505
Lactococcus lactis ssp. *lactis bv. diacetylactis* str. LD61	2.6	35	2,751	2,601
Lactococcus lactis ssp. *lactis bv. diacetylactis* str. TIFN2	2.51	35.1	2,665	2,521
Lactococcus lactis ssp. *lactis bv. diacetylactis* str. TIFN4	2.55	35	2,736	2,598
Lactococcus lactis ssp. *lactis* A12	2.7	35.3	2,887	2,725

(continued)

Table 3.2 (continued)

Strain	Size (Mb)	GC%	Gene	Protein
Lactococcus lactis ssp. *lactis* Dephy 1	2.6	35.1	2,744	2,686
Lactococcus lactis ssp. *lactis* NCDO 2118	2.81	35	–	–
Lactococcus lactis ssp. *lactis* YF11	2.53	34.9	–	–
Leuconostoc carnosum KCTC 3525	3.23	40.9	–	–
Leuconostoc gelidum KCTC 3527	1.96	36.6	–	–
Leuconostoc mesenteroides ssp. *cremoris* ATCC 19254	1.74	37.9	1,903	1,847
Leuconostoc mesenteroides ssp. *cremoris* TIFN8	1.71	38.2	1,742	1,452
Leuconostoc citreum LBAE C10	1.93	38.7	2,024	1,971
Leuconostoc citreum LBAE C11	1.97	38.6	2,089	2,036
Leuconostoc citreum LBAE E16	1.8	38.9	1,908	1,854
Oenococcus oeni ATCC BAA-1163	1.75	37.9	1,678	1,398
Oenococcus oeni AWRIB202	1.84	37.6	1,831	1,732
Oenococcus oeni AWRIB304	1.85	37.9	1,844	1,743
Oenococcus oeni AWRIB318	1.81	37.9	1,798	1,698
Streptococcus thermophilus CNCM I-1630	1.62	39.1	1,937	1,891
Streptococcus thermophilus MTCC 5460	1.61	39.3	1,764	1,706
Streptococcus thermophilus MTCC 5461	1.62	39.3	1,805	1,739
Pediococcus pentosaceus IE-3	1.8	37.2	1,762	1,710
Weissella confusa LBAE C39-2	2.28	44.7	2,237	2,156
Weissella koreensis KCTC 3621	1.73	35.5	1,750	1,672
Weissella cibaria KACC 11862	2.32	45.1	–	–
Weissella paramesenteroides ATCC 33313	1.98	37.9	2,020	1,952
Weissella halotolerans DSM 20190	1.36	43	–	–
Weissella ceti NC36	1.35	40.8	1,342	1,258

[a] Genome information is extracted from NCBI website (http://www.ncbi.nlm.nih.gov/genome/genomes/)

free-living organism, is relatively small (Vogel et al. 2011), and the *L. iners* genome has a size of 1.304 Mbp (Macklaim et al. 2010). However, surprisingly, both genomes contain an intriguingly high number of genes for stable RNAs. The in silico analysis of the genomes identified 7 and 6 rRNA operons in *L. sanfranciscensis* and *L. iners*, respectively. The enrichment of these elements in the genomes was postulated to provide a competitive mechanism for responding to environmental pressures and resource availability (Macklaim et al. 2010). The overall GC content of most LAB is less than 50 %, with the exception of *L. fermentum*, which has the highest among the sequenced LAB strains. Compared with *L. reuteri*, a phylogenetically close strain, these phenomena were explained by the high GC content at the third codon position (32 vs. 65 %) (Morita et al. 2008), and a low GC content at the third codon has been reported for *L. acidophilus* (25 %) and *L. johnsonii* (24.4 %) (Altermann et al. 2005; Pridmore et al. 2004).

3.3 Comparative Genomics

3.3.1 Genome Synteny

Synteny refers to the physical co-localisation of genetic loci on the same chromosome within an individual or species. In the current view of genetics, synteny describes the conservation of blocks of orders within two sets of chromosomes. The general finding in synteny analysis during comparative genomics can used to infer the phylogenetic relationships among species. The rapid development of bioinformatics tools can aid the exploration of the synteny features of sequenced LAB genomes (Siezen et al. 2004).

The first synteny analysis of LAB was conducted by the comparison of the genomes of *L. plantarum* and *L. johnsonii* (Boekhorst et al. 2004). At the protein level, their clear distance was confirmed, i.e. only 28 regions, ranging in size from 7 to 75 genes, showed conservation of the gene order but are not co-linear, indicating major chromosomal rearrangements (Boekhorst et al. 2004). A detailed analysis revealed that the clusters of orthologous genes conserved are located near the diagonals between the origin and the terminus of replication and show a weak X-alignment pattern (Boekhorst et al. 2004). According to Boekhorst et al. (2004), this observation indicates multiple chromosomal inversions pivoted on the terminus and origin of replication, resulting in major rearrangements. However, there is some evidence suggesting that this phenomenon is often caused by recombination events occurring close to replication forks (Boekhorst 2004; Tillier and Collins 2000).

To gain some insights into interspecies and intraspecies differences, a set of Lactobacilli consisting of thirteen strains was subjected to further comparative analysis. As depicted in Fig. 3.1 (Zhang et al. unpublished data), the above observation that a lack of extensive conservation is strong was found through the whole genome alignment of *L. johnsonii*, *L. delbrueckii*, *L. helveticus*, *L. acidophilus* and *L. gasseri*, which show a high degree of synteny and conservation over the whole genome. According to Salvetti et al. (2012), these species are included in the *L. delbrueckii* phylogenetic group composition; thus, the degree of synteny can be related to the phylogenetic distance between the organisms. A consistent phylogenetic pattern between these species can be observed from the topological structure established from their 16S RNA genes (Fig. 3.2). Syyama and Bork noted that closer genomes have a more distanced X-alignment than more distant genomes (Suyama 2001). From this point of view, the genomics-supported *Lactobacillus* genus concept is associated with the demonstration that bacteria classified outside the genus *Lactobacillus* share less synteny relatedness. Unexpectedly, in a PROmer analysis, *L. johnsonii* was found to be as closely related to *L. brevis* as to *Pediococcus*, whereas *Oenococcus* and *Leuconostoc* share practically no synteny with *L. johnsonii* (Berger et al. 2007); in addition, the genus *Lactobacillus* is paraphyletic and intermixed with *Pediococcus* species (Felis and Dellaglio 2007).

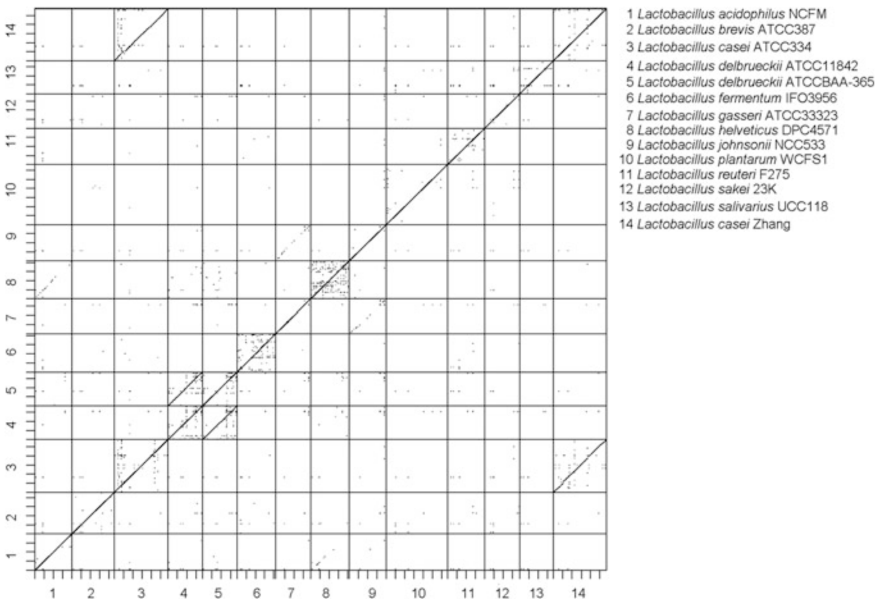


Fig. 3.1 Synteny between *L. casei* Zhang and other thirteen genomes of *Lactobacillus* (*L. acidophilus* NCFM, *L. brevis* ATCC367, *L. casei* ATCC334, *L. delbrueckii* subsp. *bulgaricus* ATCC11842, *L. debrueckii* subsp. *bulgaricus* ATCC BAA-365, *L. fermentum* IFO 3956, *L. gasseri* ATCC 33323, *L. helveticus* DPC4571, *L. johnsonii* NCC533, *L. plantarum* WCFS1, *L. reuteri* F275, *L. sakei* 23k, *L. salivarius* UCC118 and *L. casei* Zhang (Zhang et al. unpublished data))

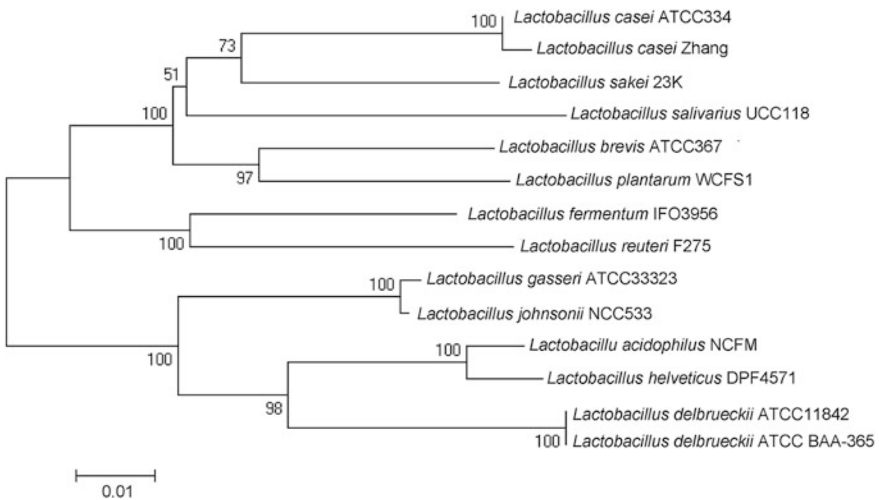


Fig. 3.2 Phylogenetic tree based on 16S rRNA gene from *L. acidophilus* NCFM, *L. brevis* ATCC367, *L. casei* ATCC334, *L. delbrueckii* subsp. *bulgaricus* ATCC11842, *L. debrueckii* subsp. *bulgaricus* ATCC BAA-36, *L. fermentum* IFO 3956, *L. gasseri* ATCC 33323, *L. helveticus* DPC4571, *L. johnsonii* NCC533, *L. plantarum* WCFS1, *L. reuteri* F275, *L. sakei* 23k, *L. salivarius* UCC118 and *L. casei* Zhang (Zhang et al. unpublished data)

3.3.2 Evolution and Biodiversity of Genomes

In the evolutionary history of LAB, gene loss and metabolic simplification have played central roles (Makarova et al. 2006). Many changes within the evolutionary phase are seemingly related to the adaptation to nutrient-rich environments (Makarova and Koonin 2007). After divergence from their ancestor, i.e. *Bacillus*, an estimated loss of 600–1,200 genes, particularly genes associated with the biosynthesis of cofactors, is predicated from the origin of *Lactobacillales* (Makarova and Koonin 2007). For example, the common ancestor of *Lactobacillaceae* and *Leuconostocaceae* lost genes for serine and glycine biosynthesis, and *L. brevis*, *Pediococcus pentosaceus*, *O. oeni*, *L. casei* and *L. delbrueckii* lost genes for arginine and aromatic amino acid biosynthesis. Moreover, many genes for sporulation, catalase and oxidative stress response were also lost. The enhanced stress response of some Lactobacilli from vacuum-dried and irradiated foods was therefore ascribed to the low content of iron and other antioxidants (Makarova et al. 2006). At this stage, negative selection pressure under an industrial environment may induce the slowing down of the process of genome decay (Hao et al. 2011).

As observed in many other bacteria, evolutionary events never occur alone. Along with prevailing gene losses, a considerable number of lineage-specific acquisitions via duplications and horizontal gene transfer (HGT) have been revealed. In the early evolutionary phase of *Lactobacillales*, those genes for phosphoenolpyruvate phosphotransferase systems (PTS), the GpmB family of sugar phosphatases, galactose mutarotase, L-lactate dehydrogenases and some peptidases were clearly duplicated (Makarova and Koonin 2007). In certain species, such as *L. iners*, the acquirement of a striking number of genes from their outside genus likely performed by HGT, and this accounts for 5.5 % of the protein-coding genes. Another interesting case for HGT is associated with genes encoding sugar uptake and utilisation. A 213-kb region from 3,072,500 to 3,285,500 in the chromosome of *L. plantarum* encodes most proteins required for sugar transport, metabolism and regulation (Kleerebezem et al. 2003). Similarly, a possible relationship between the flexibility of sugar utilisation and HGT was established in *L. casei* (Zhang et al. 2010).

Among the 80 LAB genomes of 28 recognised species that have been fully sequenced, *L. casei*, *L. plantarum*, *L. sakei*, *Lactoc. lactis*, *L. helveticus*, *S. thermophilus* and *O. oeni* were selected for further exploration of the gene content and genome evolution. Starting with *S. thermophilus*, a comparative genome hybridisation (CGH) of 47 dairy isolates was conducted for interspecies genomic comparisons. By analysing the CGH data, the first industrial core genome, which consists of 1,271 genes, was defined. The phylogenetic tree constructed showed that only a few strains can be clustered, indicating a high frequency of recombination or gene transfer in *S. thermophilus* strains (Rasmussen et al. 2008). Employing the same molecular technique, a high diversity in response to different ecological niches was confirmed in *L. sakei*, *Lactoc.lactis*, and *L. helveticus*

(Siezen et al. 2011; Kaleta et al. 2009; Nyquist et al. 2011). In particular, the genomic divergence in *L. sakei* mainly corresponded to five regions, which is in consistent with typical HGT features (Nyquist et al. 2011). Conversely, the sequence analysis of some *L. casei* isolates support the hypothesis that gene decay contributes to their enhanced fitness in cheese-like niches (Broadbent et al. 2012).

The whole genomes of 146 type strains, which represent 90 % of the species in the genus *Lactobacillus*, are going to be released soon to the public database (Prof. Zhang, personal communication). An analysis of these genomes demonstrated that the phylogeny of the genus *Lactobacillus* comprises two main branches with different evolutionary patterns. The type strains isolated from humans and animals appear to undergo the niche shift events on Branch 1 but evolve in parallel with the strains in other niches on Branch 2. Eighty-four per cent of the animal-isolated strains (36 of 43) clustered within three groups: 17 in the *L. delbrueckii* phylogroup, 10 in *L. salivarius*, and 9 in *L. reuteri*. Within each phylogroup, the animal-isolated strains had only small genetic distances from each other, suggesting that they were separately descendent from the common ancestor of each phylogroup before spreading and colonising within physically close niches, such as intestine and blood. Since the distribution of the animal-isolated strains displayed different patterns between the two main branches of the lactobacilli phylogeny, Branch 1 was split into three sections. Each section contains similar number of strains, and they were named the old, intermediate or young lineage, according to their branch order from the most recent common ancestor. Most of the animal isolates were observed in the young lineage, corresponding to the *L. delbrueckii* phylogroup. There were no animal isolates observed in the intermediate lineage, and only three were present in the old lineage. Therefore, it was proposed that colonisation ability of most of the animal isolates in Branch 1 appeared to have developed from an ancestor living in other niches. Concerning the observation of affluent niche types present in the old lineage and that the majority of the strains in the intermediate lineage being plant-associated (from the niche type plant and sourdough), it was hypothesised that the possible evolutionary scenario in Branch 1 was that the ancestor of the lactobacilli had the ability to occupy multiple types of habitats, including animal body. Then, some of the lineages evolved to fit into specific niches, most likely sourdough or other plant fermentation products. Finally, the ability to survive in an animal niche was acquired by the *L. delbrueckii* phylogroup, which evolved from plant-associated species. Their analysis also demonstrates that facultatively hetero-fermentative strains from *Lactobacillus* are the ancestors of obligately homofermentative and obligately hetero-fermentative species. After the formation of the genus, the phenotypes of obligately homofermentative and obligately hetero-fermentative were generated during the evolutionary history as a result of the variation occurring in some metabolic pathways, which resulted in the lost of homofermentative or hetero-fermentative ability. These results provide some insights into the genetic diversity of the genus *Lactobacillus* and suggest that its complexity may have been caused by various selection pressures from its multiple ecological niches.

3.4 Metabolic and Biosynthetic Capabilities

Historically, the exploration of the LAB metabolic characteristics was associated with the preservation of foods. The domestic cultivation of LAB strains was realised in the production of conventional food stuffs and has been passed down generation to generation until today. One important characteristic of LAB is their diverse capacities to utilise carbohydrates, which is reflected by the high number of genes encoding putative sugar transporters. The main components for the catalysis of sugar transport and phosphorylation are the predicted phosphoenol-pyruvate-dependent sugar PTSs (Postma et al. 1993). In addition, LAB genomes encode abundant transporter genes that are involved in the transport of carbon sources.

A broad range of variation in the number of PTS-related genes has been reported in LAB (Table 3.3) (Zhang et al. 2013). In some species, such as *L. reuteri* and *L. brevis*, which exhibit a limited capacity for carbohydrate utilisation compared with other lactobacilli, no complete EII complex was found (Zhang et al. 2013). A low number of PTS genes was also detected in *L. fermentum*, *L. bulgaricus*, *L. helveticus* and *S. thermophilus*, which are specialised to certain environmental niches (Zhang et al. 2013). On the contrary, three phylogenetically closed species, namely *L. plantarum*, *L. casei* and *L. rhamnosus*, encode a significantly higher number of PTS genes (Kleerebezem et al. 2003; Cai et al. 2009; Morita et al. 2009), i.e. more than 20 complete PTS and several incomplete PTS for mono-, di-, and polysaccharides were identified in these genomes, supporting the finding that strains from these species are capable of inhabiting various ecological niches. Similarly to *L. plantarum* (Kleerebezem et al. 2003), in many cases, the PTS enzyme II genes are clustered with glycosyl hydrolase genes and transcriptional regulator genes, suggesting a localised transcriptional control (Morita et al. 2009).

The feature of variation was also explored in a single species. Our preliminary analysis indicated that the number of PTS-related proteins, particularly the PTS enzyme II genes for fructose and mannose family, significantly varied in three completed *L. casei* genomes (Zhang et al. 2013). In contrast, genes encoding PTS systems are highly conserved in *O. oeni* strains (Jamal et al. 2013). *L. casei* is a versatile microorganism that can be found in various niches, whereas *O. oeni* is indigenous to wine or similar niches. Thus, the flexible ability for sugar utilisation by these two different species can be easily ascribed to their adaptation to different ecological niches.

Once internalised, LAB often employ homo- or hetero-fermentative fermentation using hexoses. The primary end products of the former pathway mainly consist of lactic acid, whereas the latter produces lactic acid, CO_2, acetic acid and sometimes ethanol (Kandler 1983). Not surprisingly, their genome information confirmed that the energy metabolism of LAB mainly relies on anaerobic glycolysis or the phosphoketolase pathway (PKP). The complete PKP pathway was found to be encoded by all LAB cocci that have been published, although most are

Table 3.3 PTSs in lactic acid bacterial strains [adapted from Zhang et al. (2013)]

Strains	PTS EII complex family							Hpr-EI	Total PTS	PTS related proteins
	Glucose	Fructose	Lactose	Glucitol	Galactitol	Mannose	Ascorbate			
Lactobacillus acidophilus NCFM	6	0	1	0	0	0	0	Y	7	31
Lactobacillus brevis ATCC367	0	0	0	0	0	0	0	Y	0	8
Lactobacillus casei ATCC334	5	2	1	1	2	4	2	Y	17	67
Lactobacillus casei BL23	4	5	6	1	3	11	3	Y	33	122
Lactobacillus casei Zhang	6	6	5	1	4	6	3	Y	31	96
Lactobacillus bulgaricus ATCC11842	0	1	0	0	0	1	0	Y	2	14
Lactobacillus bulgaricus ATCCBAA-365	0	1	0	0	0	1	0	Y	2	11
Lactobacillus fermentum IFO3956	1	0	0	0	0	1	0	Y	2	10
Lactobacillus gasseri ATCC33323	6	2	3	0	1	3	0	Y	15	40
Lactobacillus helveticus DPC4571	1	1	0	0	0	1	0	Y	3	15
Lactobacillus johnsonii NCC533	7	2	1	0	1	5	0	Y	16	47
Lactobacillus plantarum WCFS1	14	3	2	2	2	2	1	Y	26	72
Lactobacillus reuteri F275	0	0	0	0	0	0	0	Y	0	7
Lactobacillus sakei 23 K	3	1	0	0	0	1	0	Y	5	18
Lactobacillus salivarius UCC118	2	2	0	0	0	3	0	Y	7	20
Lactococcus lactis ssp. *cremoris* MG1363	3	2	1	0	0	1	1	Y	8	21
Lactococcus lactis ssp. *cremoris* SK11	2	2	1	0	0	1	1	Y	7	18
Lactococcus lactis ssp. *lactis* Il1403	2	1	1	0	0	1	0	Y	5	18
Streptococcus thermophilus CNRZ1066	1	0	0	0	0	1	0	Y	2	22
Streptococcus thermophilus LMD-9	1	0	0	0	0	1	0	Y	2	9
Streptococcus thermophilus LMG18311	1	0	0	0	0	1	0	Y	2	22

O. oeni, S. thermophilus and *Leuconostoc* strains (Borneman et al. 2012). In Lactobacilli, it is believed that glucose metabolism is the primary catabolic pathway. However, two essential genes, namely the *pfk* gene encoding 6-phosphofructokinase and the *fba* gene encoding fructose-bisphosphate aldolase, are usually not found in *L. reuteri* JCM 1112^{T}, *L. fermentum* IFO 3956 and *L. brevis* are thus proposed to be key genes for distinguishing homo- and hetero-fermentation (Morita et al. 2008). The presence of the complete EMP pathway in *L. plantarum*, *L. acidophilus*, *L. sakei*, *L. casei*, *Lactoc. lactis*, *L. helveticus*, *L. gasseri*, *L. johnsonii*, and *L. bulgaricus* is in agreement with their classification as homofermentative bacteria. One exception was found with *L. salivarius*, which was regarded as homofermentative and possesses a functional PKP (Claesson et al. 2006).

In addition to the common pathways, the catabolism of several unusual carbohydrates, including pullulan, fructooligosaccharide, raffinose and *myo*-inositol (MI), was discovered and identified in LAB (Goh and Klaenhammer 2009; Barrangou et al. 2003; Goh et al. 2006; Saulnier et al. 2007; Yebra et al. 2007). For example, the genes associated with MI utilisation are encoded by an operon consisting of the EeoR family transcriptional repressor (*iolR*) and a divergently transcribed *iolTABCED1G2EJK*, which is regulated by substrate-specific induction mediated through the inactivation of *iolR* and by carbon catabolite repression mediated through the catabolite control protein A (Yebra et al. 2007). A comparative analysis showed that the *iol* clusters harboured by *L. casei* members, namely *L. casei*, *L. paracasei* and *L. rhamnosus*, show high similarity with the *iol* clusters of *Bacillus subtilis* but significantly differed from that of *L. plantarum* (Zhang et al. 2013). To expand our understanding of its initial origin, we performed phylogenetic studies (Zhang et al. unpublished data). Representative *iol* gene homologues in the present database were retrieved for tree reconstruction. The phylogenetic trees constructed using the minimum-evolution and neighbour-joining methods showed highly consistent topology. The analysis of their efficiencies showed that *iolD* and *iolC* tend to be better for obtaining informative evolutionary inference. In particular, most genes in the *iolD* trees can be clustered into well-defined clades supported by high bootstrap values of more than 60 %. As depicted in Figs. 3.3 and 3.4, the *iol* genes of *L. casei* members are located in one clade in either of the trees, and similarly to the findings from 16S rRNA analysis, *L. rhamnosus* was a slightly distant subspecies. The *L. casei* group is always found close to Bacillus from Firmicutes and shows a weak relationship with its *Lactobacillus* counterparts from the *L. plantarum iol* cluster. Taxonomically, the genus *Lactobacillus* and Bacillus are recognised within the same order *Lactobacillales*. In fact, a small number of *Lactobacillus*, including some *Lactobacillales*, strains have been detected to contain a homologous *iol* segment, as was also found in the previous analysis. Therefore, we postulate that the *L. casei iol* cluster was acquired by ancestral *L. casei* members via HGT. In addition, because most subspecies of the *L. casei* group exhibit the MI pathway, we hypothesised that this event most likely occurred before the subspecies divergence of *L. casei* group.

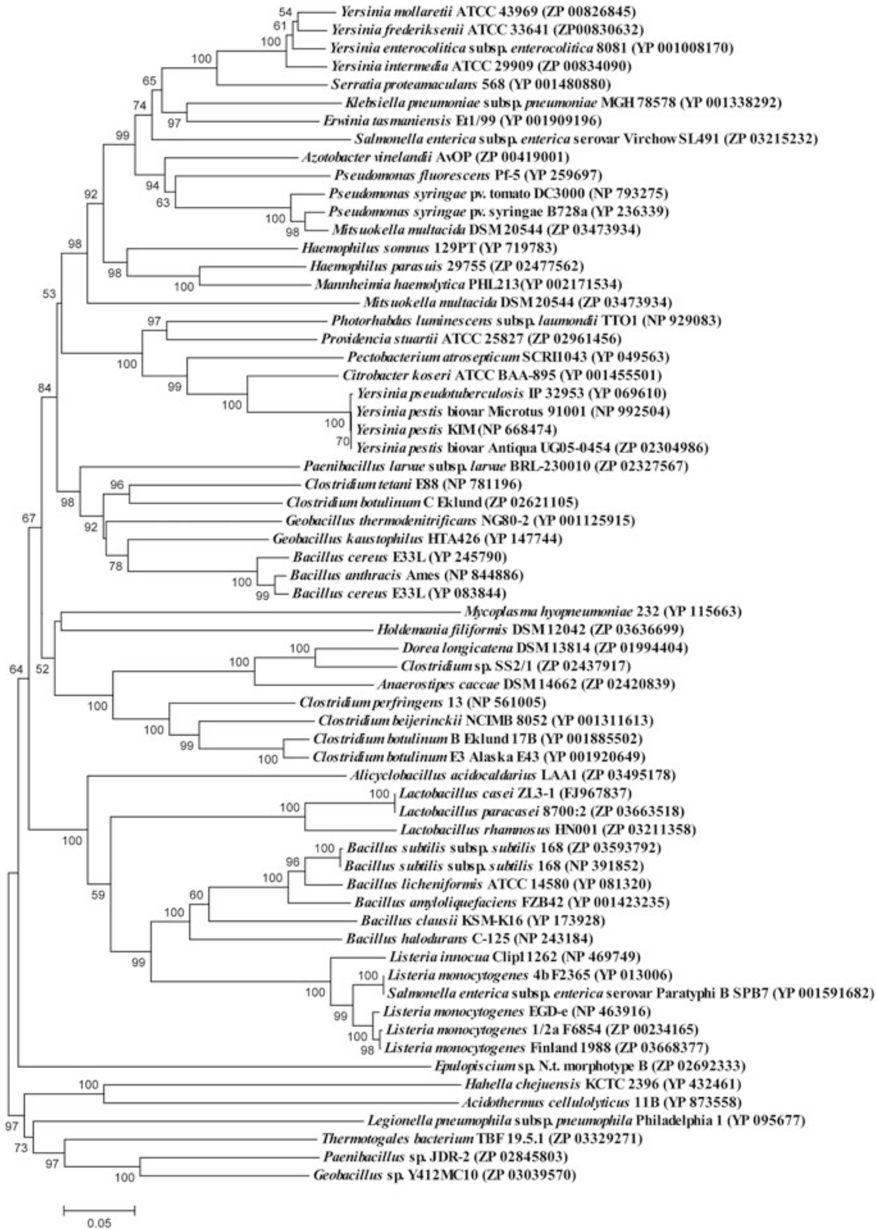


Fig. 3.3 Phylogenetic reconstruction of *iolD* genes (Zhang et al. unpublished data)

It is well known that LAB are fastidious in their nutritional requirements and that that this can be mirrored by the development of minimal growth medium (Reuter 1985). The addition of some cofactors and vitamins is somewhat required

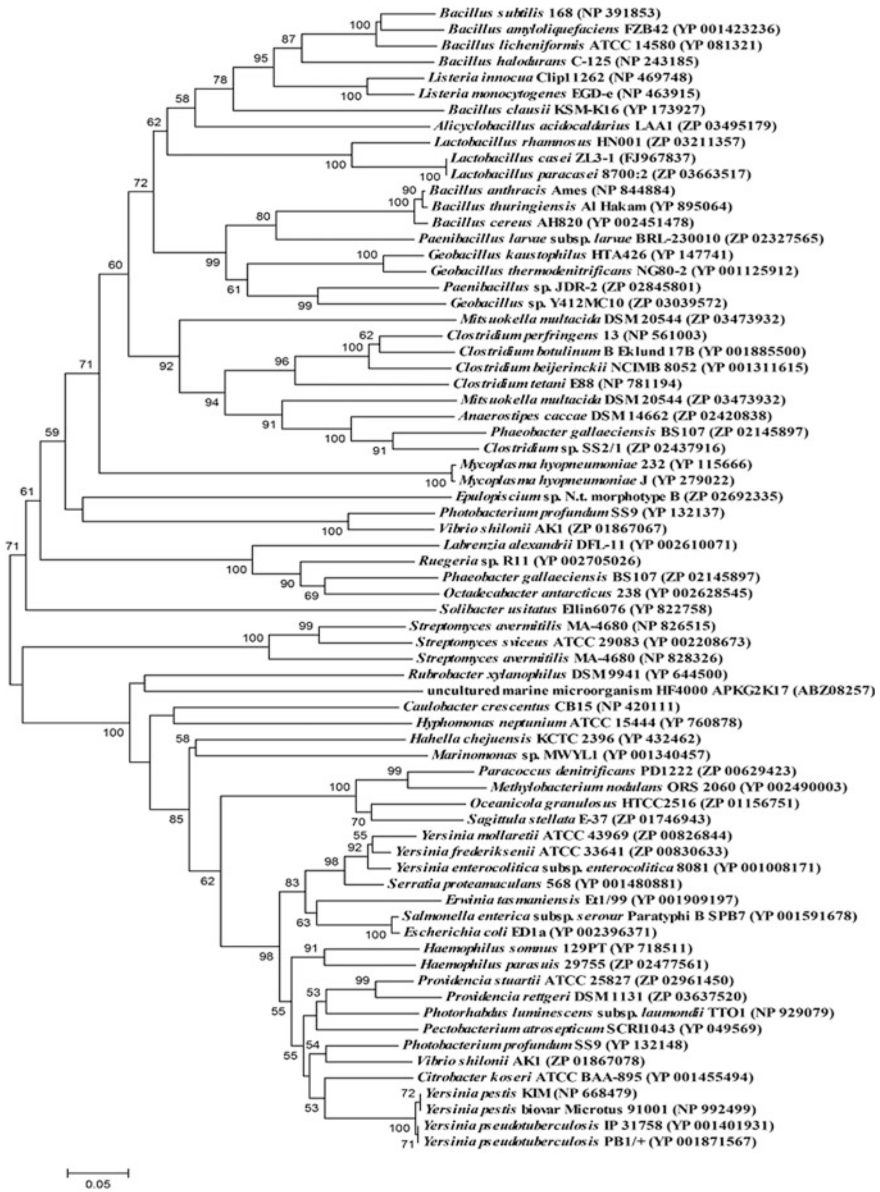


Fig. 3.4 Phylogenetic reconstruction of *iolC* genes (Zhang et al. unpublished data)

(Hugenholtz and Kleerebezem 1999). Due to a lack of key enzymes, LAB have a limited capacity to synthesise these components. Unlike *L. johnsonii*, *L. acidophilus*, *L. gasseri* and many other Lactobacilli, *L. iners* appears to have the genetic potential to synthesis folate because a cluster of the associated enzymes

were found in the genome (Macklaim et al. 2010), but this hypothesis needs to be further studied. The analysis of the synthesis of reuterin and cobalamin (vitamin B_{12}) revealed that the 58-gene cluster in *L. reuteri* is located in a genomic island and inserted into the conserved region (Morita et al. 2008). Similarly, *Lactoc. lactis* presents a complete pathway for riboflavin synthesis, and the ability to excrete small amounts of the compound has been reported (Hugenholtz et al. 2002).

Another important growth stimulation factor for LAB is amino acids; however, their biosynthetic pathways showed some deficiencies. With the exception of two versatile species, namely *L. plantarum* and *L. casei*, which are only auxotrophic for leucine, isoleucine and valine, most LAB species lack the de novo synthetic capability for multiple amino acids (Cai et al. 2009; Schroeter and Klaenhammer 2009). Based on some newly published genomes, the auxotrophy level could be extended further (Vogel et al. 2011; Forde et al. 2011). *L. iners*, the most frequently detected bacterial species in the vagina, is unable to synthetic any amino acids de novo. Instead, this species synthesises serine, L-aspartate, L-asparagine, and glutamine by interconversion via existing key enzymes (Macklaim et al. 2010). Notably, it has been shown that the absence of some biosynthetic capabilities is associated with adaptations to the environment. *O. oeni* lost the biosynthetic capabilities for proline and serine that must be present in wine but retains the ability to synthesise cysteine and methionine, which are known to be present in wine at low concentrations (Mills et al. 2005). Furthermore, close relationship between amino acid production and health-promoting effects is also established. A strain of *Weissella koreensis* designated OK1-6 was reported to produce ornithine from arginine, which implies its functional role in reducing obesity (Moon et al. 2012).

To compensate for the extensive loss of biosynthetic pathways for various amino acids, LAB are generally equipped with a protein degradation-machinery to acquire and utilise exogenous nitrogen sources, which may provide some competitive advantage in protein-rich environments (Kok 1990). The so-called proteolytic system typically consists of cell-wall proteinases, transport systems and peptidases. Under these conditions, the proteinases will initiate the first step of the reaction for the degradation of extracellular proteins into oligopeptides (Mayo 1993). Within LAB genomes, a large diversity was observed in terms of the proteolysis-related gene content. A relatively high number and variety of proteolytic system components were identified in the *L. acidophilus* group, which are composed of *L. acidophilus*, *L. johnsonii*, *L. gasseri*, *L. bulgaricus* and *L. helveticus* strains. In particular, the cell-wall-bound proteinase (*PrtP*) was only found on the chromosomes of *L. acidophilus*, *L. johnsonii*, *L. bulgaricus*, *L. casei*, *L. rhamnosus* and *S. thermophilus* (Liu et al. 2010). In addition, many of the peptidases, such as the aminopeptidase *PepC*, *PepN*, and *PepM* and the proline peptidases *PepX* and *PepQ*, which are essential for bacterial growth, were found in all genomes (Liu et al. 2010).

3.5 Regulation

To survive in various environments, LAB have to modulate their gene expression repertoire at all times. Industrialised LAB would have to survive production processes, the gastrointestinal (GI) tract of human or animals and acidic and alcoholic food formulations. The adaptive responses to these environments are usually mediated by two-component systems (TCSs) comprising a sensor histidine kinase (HK) and a response regulator (RR). The role of HK is to monitor the environmental signals and respond through autophosphorylation and the transfer of a phosphoryl group to its cognate RR. The activity of RR is therefore regulated by phosphorylation (Chang and Stewart 1998).

The *L. casei* genomes contain 16/17 TCSs, which is the highest number among the sequenced LAB (Zhang et al. 2013). In *L. sanfranciscensis, Lactoc. lactis*, *S. thermophilus, L. delbrueckii* ssp. *bulgaricus*, *L. sakei*, *L. gasseri*, *L. johnsonii, L. acidophilus*, *L. iners*, *L. helveticus* and *L. plantarum*, 2, 7, 9, 5, 10, 4, 10, 9, 4, 9 and 13 TCSs were identified, respectively (Cai et al. 2009; Chaillou et al. 2005; Linares et al. 2010; Hols et al. 2005). The uneven distribution of TCSs in these species was explained by the different demands of their adaptive regulation required in their diverse niches (Cai et al. 2009). Overall, a lower number of TCSs were identified in the species that are frequently found in nutrient-rich environments. Notably, a markedly low number of genes involved in TCSs were found in *L. sanfranciscensis* compared with other LAB. This species accounts for more than 90 % of the microflora in traditional fermented sourdoughs. The low abundance of TCSs in *L. sanfranciscensis* may support the hypothesis that less adaptive regulation is required for adaptation to a stable and nutrient-rich environment (Vogel et al. 2011).

A number of the TCSs mentioned above have been identified. The possible roles of the TCSs, including the TCSs from *L. plantarum*, *Lactoc. lactis*, *L. sakei* and *L. casei* (Pfeiler et al. 2007; O'Connell-Motherway et al. 2000; Morel-Deville et al. 1998; Sturme et al. 2005; Alcantara et al. 2010), are widely correlated to quorum sensing and the production of bacteriocins (Alcantara et al. 2010). In fact, some of the TCSs appear to be involved in certain metabolic pathways. It is known that *L. casei* can metabolise L-malic acid via malolactic enzyme or malic enzyme. The key enzymes for the utilisation of L-malic acid and a complete TCS are organised into two diverging operons in the genomes of *L. casei*. It has been shown that the deletion of either TCS component would result in the loss of the ability to utilise L-malic acid, suggesting the essential roles of cognate TCS regulators (Landete et al. 2009). In addition, a group of TCSs were found to be involved in the response against antimicrobial peptides. In addition to the possession of an intramembrane-sensing kinase, these are located next to ABC transporters of the peptide-7 exporter family (Revilla-Guarinos et al. 2013).

The association of TCSs with the acid and bile stress response was simultaneously described in a probiotic *L. acidophilus* strain. A 2CRS from this bacterium similar to the *lisRK* system, which participates in both stress response and

virulence in *Listeria monocytogenes*, was disrupted to investigate its putative role in acid tolerance (Azcarate-Peril et al. 2005). Using a whole-genome microarray containing 97.4 % of the annotated genes, the transcription patterns at various pH values of the control and the histidine protein kinase mutant are significantly different from each other (Azcarate-Peril et al. 2005). The expression pattern of approximately 80 genes appeared to be either indirectly or directly regulated by the kinase. As expected, the mutant exhibited lower tolerance to acid in the log-phase of growth and poor acidification rates in milk (Azcarate-Peril et al. 2005). Using the same strategy, a TCS from this *L. acidophilus* strain located within a 7-kb eight-gene operon that includes a transporter, an oxidoreductase, and four hypothetical proteins was demonstrated to play a role in bile tolerance and bile sensitivity. The mutated RR showed enhanced induction of the operon in response to bile (Pfeiler et al. 2007).

3.6 Cell Surface Factors

LAB are of interest partly due to their major contribution to human GI ecology. The amounts of LAB members are variable throughout the GI tract, i.e. approximately 1 % microorganisms per gram of luminal content in the distal gut and 0.01 % of the total culturable counts from faces (Dal Bello et al. 2003). In the proximal small intestine, which benefits from the carbohydrates provided by the host diet, these organisms are among the dominant bacteria (Bongaerts and Severijnen 2001). Various cell surface factors are believed to be important during the interactions of LAB with their host environment through their participation in pathogen exclusion and immunomodulatory activities (Boekhorst et al. 2006). In addition to the predicted versatility of their secretome (Boekhorst et al. 2006), adherence factors have been extensively identified and functionally characterised in the sequenced LAB strains (Table 3.4).

A high-molecular-mass cell-surface protein that adheres to mucus components in vitro was first reported in *L. reuteri* 1063 (Roos and Jonsson 2002). The amino acid sequence of the protein exhibits the domain organisation similar to that of cell-surface proteins of Gram-positive bacteria, the typical architecture of which includes a signal peptide, C-terminal anchoring sequences and repeated regions (Navarre and Schneewind 1999). In silico sequence analysis also detected two possible translation sites in the *mub* genes (Roos and Jonsson 2002). Similar to the 49-aa signal peptide, the second site is proposed to generate a 35-aa secretion signal peptide with the same predicted cleavage site. A stem-loop structure known to be required for many programmed frameshifts was found after the second start site (Roos and Jonsson 2002). The use of bioinformatic tools revealed that the distribution of MUB-domain-containing proteins has a strong bias towards lactobacilli that are GI tract, i.e. *L. johnsonii*, *L. acidophilus,* and *L. gasseri* contain more than nine MUB proteins in their genomes (Boekhorst et al. 2006). Compared with these species, *L. reuteri*, *L. brevis*, *L. fermentum*, *P. pentosaceus* and *Lactoc.*

Table 3.4 Functional characterised adhesion genes in LAB

Strains	Gene name	Target for binding	Domain[a]	Accession number	References
Lactobacillur reuteri 1063	*Mub*	Mucus components	LPXTG (PF00746) MucBP (PF06458) YSIRK (PF04650)	AAF25576	Roos and Jonsson (2002)
Lactobacillur reuteri 104R	*MapA*	Caco-2 cells and mucus	Bacterial extracellular solute-binding domain (PF00497)	CAC05301	Miyoshi et al. (2006)
Lactobacilus brevis ATCC 8287	*SlpA*	Human intestinal epithelial cell lines, laminin, collagen and fibronectin	–	CAA78618	de Leeuw et al. (2006)
Lactobacillus acidophilus NCFM	*fbpA*	Human intestinal epithelial cell lines	Fibronectin-binding domain (PF05833) Domain of unknown function (PF05670)	AAV42987	Buck et al. (2005)
	SlpA	Human intestinal epithelial cell lines	MucBP (PF06458)	AAV42987	Buck et al. (2005)
	Mub	Human epithelial cell lines and mucus	MucBP (PF06458) YSIRK (PF04650) Gram-positive anchor LPxTG (PF00746)	AAV42987	Buck et al. (2005)
Lactobacillus casei BL23	*fbpA*	Human intestinal epithelial cell lines	Fibronectin-binding domain (PF05833) Domain of unknown function (PF05670)	FM177140	Munoz-Provencio et al. (2009)
Lactobacillus rhamnosus GG	–	Human intestinal mucus	MucBP (PF06458)	FM179322	von Ossowski et al. (2011)
	SpaC	Human intestinal mucus	Willebrand factor, type A (PF00092) Collagen-binding surface protein Cna-like, B-type domain (PF05738)	FM179322	Kankainen et al. (2009)

(continued)

Table 3.4 (continued)

Strains	Gene name	Target for binding	Domain[a]	Accession number	References
Lactobacillus salivarius UCC118	*LspA*	Human epithelial cell lines	Gram-positive anchor LPQTG (PF00746) MucBP (PF06458)	NC_007929.1	van Pijkeren et al. (2006)
Lactobacillus plantarum WCFS1	*Msa*	Mucus via mannose binding	Legume lectin domain (PF00139) MucBP (PF06458)	NC_004567.2	Pretzer et al. (2005)
Lactobacillus plantarum LM_3	*EnoA1*	Human fibronectin	–	–	Castaldo et al. (2009)
	pdhB	Human fibronectin	–	–	Vastano et al. (2013)
Lactobacillus johnsonii NC533	*EF-Tu*	Human intestinal cell lines and mucus	GTP binding domain (PF00009)	NC_005362.1	Granato et al. (2004)
	GroEL	Human intestinal epithelial cells and mucus	Chaperonin family (PF00118)	NC_005362.1	Bergonzelli et al. (2006)
Lactobacillu helveticus R0052	*Slp*	Human intestinal cell lines	SLAP bacterial surface layter domain (PF03217)	AAZ99044	Johnson-Henry et al. (2007)

[a] Domain information obtained at the website of Interpro (http://www.ebi.ac.uk/interpro/)

lactis live in a more restricted habitat or are less present in the GI environment. Their narrower lifestyle may be a possible explanation for their fewer MUB proteins (approximately two) (Boekhorst et al. 2006). An exception is *L. rhamnosus* because strains of this species have been isolated from various niches. Unlike *L. plantarum*, a search of the genome predicted that only one protein exhibits homology with a known mucus-binding domain (von Ossowski et al. 2011). If the previous explanation is reasonable, the mere presence of MUB proteins in *L. rhamnosus* may suggest that the test strain *L. rhamnosus* GG should be a niche specialist stemmed from an ancestral *Lactobacillus.*

Fibronectin, a dimeric 454-kDa glycosylated protein, is found in its soluble form in plasma on the host cell surfaces. It is an important target for LAB attachment. Experimental evidence shows the attachment of LAB to fibronectin from a biochemical level, and molecular tests have been performed with strains of the genus *L. acidophilus*, *L. brevis*, *L. casei*, and *L. plantarum* (de Leeuw et al. 2006; Buck et al. 2005; Munoz-Provencio et al. 2009; Vastano et al. 2013). A surface layer protein (*slpA*) and fibronectin-binding protein (*fbpA*) are involved in fibronectin binding. As indicated by a genome search using the NCBI website, homologues of *fbpA* appear to be present in all lactobacilli (Munoz-Provencio et al. 2009). This protein from *L. acidophilus* contains the N-terminal Pfam domain (pfam05833) with relatively high amino acid identity (>65 %) to its counterparts from *L. gasseri* and *L. johnsonii* (Buck et al. 2005). The inactivation of *fbpA* in *L. acidophilus* significant decreases its adhesion to Caco-2 cells, which indicates the importance of this protein for the attachment of this strain to intestinal cell lines (Buck et al. 2005). However, some attachment to Caco-2 cells was observed with *L. casei* mutants lacking a functional version of this protein (Munoz-Provencio et al. 2009). Whether this protein functions in a different manner in *L. casei* and *L. acidophilus* needs to be further clarified.

Genes encoding pilin subunits and pilin-specific sortases have been well-characterised in Gram-positive pathogens and are also adhesion mediators in LAB. These genes were exclusively identified in the newly published genomes of two *L. rhamnosus* strains, namely GG and LC705, and no data have been reported for other LAB species (Kankainen et al. 2009). In fact, two and one pili-related gene clusters were identified in the GG and LC705 strains, respectively. Because the LC705 strain is an adjunct starter culture exhibiting reduced binding to mucus, the unique gene cluster contained genes for three secreted LPXTG-like pilins (*spaCBA*) and a pilin-dedicated sortase, and these were postulated to have a positive correlation with the adhesion capability of strain GG. Unexpectedly, the physical presence of cell-wall-bound pili was confirmed by immunoblotting using anti-Spac antibodies and was also linked to adherence to the intestinal mucus. The deletion of this gene from the chromosome clearly abolished the strains' ability to bind the mucus, which indicates that this gene plays essential roles in the interaction of GG with the human intestinal tract.

It is likely that LAB species, particularly *Lactobacillus* species, employ similar adherence mechanisms in their interactions with the intestinal environment, which is a mucus-abundant surface. Nevertheless, the increasing genome data from

isolates that are indigenous to such environments are contradictory. The genome analysis of *L. iners*, an isolate from healthy human vagina, lacks most of the known adhesion factors and conserved adhesion-related domains that are present in lactobacilli. Only one fibronectin-binding protein and one fibrinogen-binding protein were identified in the search for adhesion factors, and no mucus-binding proteins were found, suggesting that a novel mechanism is used in the vagina (Macklaim et al. 2010). *L. iners* encodes a total of 14 putative adhesion factors, including sortase-dependent proteins, LPXTG proteins, and proteins that show weak similarity to adhesions proteins. In fact, the observation that seven of these 14 putative adhesion proteins have a CAI greater than the mean CAI is in support of the assertion of a hypothetical, unknown adherence method.

3.7 Polysaccharide Biosynthetic Gene Clusters

Exopolysaccharides (EPSs) from LAB are classified into homo-polysaccharides and hetero-polysaccharides according to their composition. Their described applications are to improve the texture, mouth-feel and water retention of fermented dairy foods (Hassan 2008). The biological functions of EPSs are diverse: these products may protect cells from desiccation and other environmental stresses and adhere to solid surfaces. It is now accepted that EPSs play important roles in immune stimulation, biofilm formation and the associated infection (Hosono et al. 1997). Additional intestinal research has implied that the retention time of EPSs in the GI tract may enhance the colonisation of probiotic bacteria (German et al. 1999).

The biosynthetic pathways leading to EPS synthesis are present in most LAB species that have reported. The genetic basis typically consists of genes encoding chain length determination, repeating unit biosynthesis, polymerisation and export. The homologies shared by the genes present in the EPS clusters from *L. rhamnosus* and *L. helveticus* strains are striking, implying a common mechanism of EPS biosynthesis (Jolly and Stingele 2001; Peant et al. 2005). However, the glycosyltransferases located in the central region of the EPS clusters often show low amino acid sequence similarities, as determined using linear alignment methods. The large variety in the specificities of these enzymes can be regarded as a toolbox for their application in EPS engineering. The current challenges are the production of EPSs with a structure and size that impart the desired functionality (Welman and Maddox 2003).

The EPS cluster in *L. acidophilus* comprises 14 genes, including the proteins *EpsA–EpsF*, *EpsJ* and *EpsI* and five variable proteins representing glycosyl transferases and polysaccharide polymerases (Altermann et al. 2005). This set of genes showed high synteny to the EPS clusters in Streptococci and its closed related species *L. gasseri* and *L. johnsonii* (Altermann et al. 2005; Pridmore et al. 2004). Unfortunately, no clear evidence has demonstrated whether this cluster is functional in the corresponding strain *L. acidophilus* NCFM. Together, all three

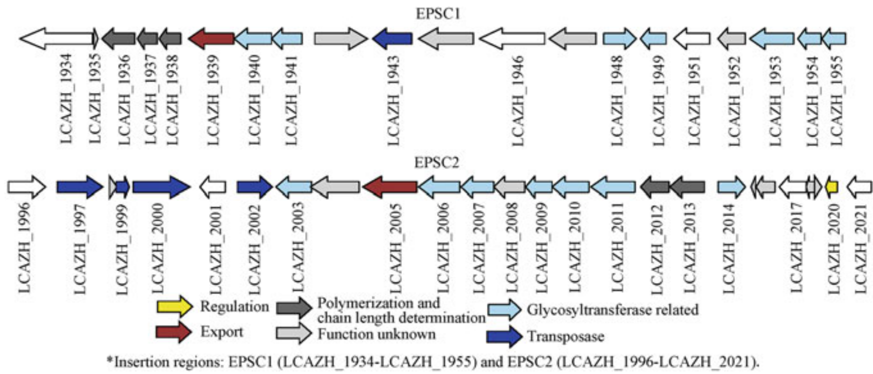


Fig. 3.5 Genetic structure of EPS cluster in *L. casei* Zhang (Generated using an in-house perl script with reference to Zhang et al. (2013)

gene clusters in the genome are located in the region with markedly low GC content, and the presence of a transpose gene in the downstream region may further indicate their acquisition via HGT (Altermann et al. 2005). Similarly, *L. ruminis* contains one EPS cluster, which incorporates 62 predicted coding sequences (Forde et al. 2011). The EPS gene cluster also contains four transposases. However, interestingly, the *L. ruminis* EPS gene cluster has an atypical G + C content relative to that of its genome: the G + C content of the EPS locus is 39.66 % compared with an approximately 44.4 % G + C content in the rest of the genome.

Unlike *L. plantarum*, *L. salivarius*, *L. sakei* and *L. bulgaricus*, *L. casei* has two EPS clusters, although these vary between different strains. As was observed in a comparative gnomic analysis of the *L. casei* strains Zhang, BL23 and ATCC334 (Zhang et al. 2013), the two EPS clusters identified in Zhang are encoding by the insertion regions detected (Fig. 3.5). EPSC1 and EPSC2 from the Zhang strain have G + C contents of 39.2 and 36.6 %, respectively, which are similar to those found in *Streptococcus* (~39 %) but different from those found in *Lactoc. lactis* (~32 %) and *L. bulgaricus* (45 %). The best hits found for the amino acid sequences deduced from the CDSs present in EPSC1 and EPSC2 ranged from 25 to 88 % (Zhang et al. 2013). EPSC1 (~27 kb) encodes 20 putative proteins, all of which are CDSs, and three were found on the lagging strand. Six glycosyltransferases and one acyltransferase were found in EPSC1 (Zhang et al. 2013). EPSC2 (~19 kb) encodes 18 putative genes. Moreover, eight genes possibly related to polysaccharide biosynthesis, including one glycosyltransferase, one alcohol dehydrogenase, one regulator and one mutase, were found and are conserved in *L. casei* ATCC334 (Zhang et al. 2013).

A significant variability in the gene contents associated with the distribution of EPS genes is also observed in *L. bulgaricus*, *Lactoc. lactis*, *S. thermophilus* and *O. oeni*. The lengths of these two neighbouring clusters in *L. bulgaricus* strain 2038 are 16 and 12 kb, respectively (Hao et al. 2011). Compared with the clusters in two

other strains, the 16-kb EPS cluster has eight unique genes, five of which encode glycosyl transferases, and the 12-kb EPS cluster also showed significant differences with respect to the gain or loss of some GTF-encoding genes. It was therefore proposed that the EPS repeat unit produced by either the 16- or the 12-kb clusters must differ from those produced by the other two strains. Siezen et al. (2011) presented a large EPS cluster of approximately 25 genes that are responsible for the formation of rhamnose-glucose polysaccharides. This EPS cluster consists of three separate parts, and the third set of genes, which is presumably involved in glycerophosphate-containing lipoteichoic acid biosynthesis, is only present in three out of 39 *Lactoc. lactis* strains. In agreement with this finding, a high degree of potential intraspecific diversity in EPS clusters was confirmed in *S. thermophilus* and *O. oeni* (Rasmussen et al. 2008; Borneman et al. 2012), suggesting a rich variety in the structures of the produced EPS that may result in different technological properties that may be of interest for the production of certain foods.

3.8 Bacteriocin Biosynthesis

The antimicrobial effect of LAB has been documented for thousands of years and enables the extension of the shelf life of fermented products. This effect is mainly due to the production of lactic acid. However, the production of antimicrobial compounds, e.g. bacteriocins known as antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria, is receiving growing attention (Ryan et al. 1996). Since the discovery of nisin, a large number of bacteriocins have been biochemically characterised. In fact, four classes, including lantibiotics, small heat-stable non-lantibiotics, large heat-labile bacteriocins and an undefined mixture of proteins, have been established (Nes et al. 1996).

Using the gene context approach, the clustered genes for putative bacteriocins and associated proteins were identified in several LAB species. According to Makarova and Koonin (2007), two families of detected candidate bacteriocins are homologs of pediocin from *P. pentosaceus* (homologs of this protein are present in *Le. mesenteroides* and *L. casei*) and homologs of divercin (homologs of this protein are present in *P. pentosaceus* and *L. johnsonii*). The bacteriocins that have been discovered to date from sourdough LAB include the bacteriocins bavaricin A plantaricin ST31 and the bacteriocin-like inhibitory substance *L. sanfranciscensis* C57; however, no active bacteriocin genes are found in the genome of *L. sanfranciscensis* TMW 1.1304 (Vogel et al. 2011). Similarly, no complete bacteriocin synthesis genes were found in the *L. inters* AB-1 genome, but three genes encoding the putative bacteriocin immunity protein of *Finegoldia magna* and proteins with greater than 60 % identity to the MccC microcin immunity family of proteins from strains of *S. pneumoniae* were found (Macklaim et al. 2010). Thus, it is possible that bacteriocin production is not responsible for the long-term competitiveness of these bacteria in special niches.

The analysis of the particular function of chromosome-encoded bacteriocin clusters should include *L. acidophilus* and *L. salivarius*. The gene cluster for the production of class II bacteriocin in *L. acidophilus* NCFM was identified in a 9.5-kb region (Altermann et al. 2005). The region contains 12 putative genes that are responsible for the production and processing of lactacin B and is organised into three clusters: a production and regulation cluster encoding a putative two-component signal transduction system, an export cluster encoding a putative ABC transporter, and a cluster composed of three unknown proteins. The deletion of a predicted ABC transporter completely abolished the bacteriocin activity, whereas its cloning and expression resulted in markedly higher levels of lactacin B activity (Dobson et al. 2007). As observed in *L. salivarius*, the production of this bacteriocin is regulated by the three-component regulatory system common to class II bacteriocins, which also includes an HK and an RR (Flynn et al. 2002).

3.9 Prophage

Having prophage sequences is a common trait of bacterial chromosomes, where they organise as a mosaic pattern and exhibit extensive horizontal genetic exchange among different members (Hendrix et al. 1999). This is also common to LAB because many published genomes harbour prophage or prophage remnants (Zhang et al. 2010), which contribute a substantial share of the mobile DNA of their bacterial hosts. In some cases, certain niche-specific genes also appear in these prophages.

Because of the economical impact in the industry for the manufacturing of fermented dairy products, the interest in phages originally arose from *Lactoc. lactis* strains. The constant exposure to dairy plants and the manufacturing processes used create a strong selective pressure on bacteria and phages. Prophages from this species therefore become an interesting model for the study of their evolution. Chopin et al. (2001) reported the genetic organisation of six prophages present in the *Lactoc. lactis* genome: the three larger prophages (36–42 kb) belong to the previously described P335 group of temperate phages, whereas the three smaller ones (13–15 kb) are most likely satellites relying on helper phages for multiplication. The analysis of the genetic structure of all known groups of phages active on other bacterial hosts further confirmed the existence of two types of genetic structures related to the phage lifecycle, which reflect the different intensities of horizontal DNA exchange. Moreover, this finding suggests that the constraints on genetic exchange among purely virulent phages reflect the optimal genetic organisation.

With respect to *Lactobacillus*, the amount of prophage sequences in *Lactobacillus* genomes is variable, ranging from one to four clusters in *L. gasseri*, *L. reuteri*, *L. brevis*, *L. rhamnosus*, *L. casei*, *L. salivarius, L. plantarum*, and *L. johnsonii* (Kankainen et al. 2009; Azcarate-Peril et al. 2008; Claesson et al. 2006). In some species, such as *L. acidophilus*, only prophage remnants were detected

(Altermann et al. 2005). However, no significant nucleotide sequence similarity was detected between phages infecting distinct bacterial species (Desiere et al. 2002). In addition, identical overall genomic organisation was detected between *L. delbrueckii* phage LL-H and *S. thermophilus* phages belonging to the proposed Sfi11-like genus of Siphoviridae. It appeared that relatively tight barriers prevented the transfer of phage genes across *L.* species. Until the publication of *L. casei* Zhang, one chromosome-encoded prophage remnant (Lcazh1) was identified within the 12.5-kb-long nucleotide sequence containing 20 coding genes. With the exception of one uncharacterised protein cluster, the genes coded by Lcazh1 share extensive similarities and strong synteny with the prophage Lp3 harboured by *L. plantarum* WCFS1, implicating the possibility of lateral DNA transfer (Zhang et al. 2010).

Bacteriophages are a common and constant threat to milk fermentation. Thus, the stability of the prophages widespread in LAB has received much attention. It has been demonstrated that the induction of LAB prophages sometimes occurs under the stress of mitomycin C or ultraviolet light (Chopin et al. 1989). For *Lactoc. lactis* host strains, the maximum spontaneous induction frequency of the prophage varies in different lysogenic strains, and there is no correlation between the growth rates of the host cells and the spontaneous prophage induction frequencies (Lunde et al. 2003). Notably, large parts of the prophage genome in *Streptococcus* and *Lactococcus* were transcriptionally silent during the lysogenic state, whereas genes encoding immunity against phage superinfection and those maintaining the lysogenic state are highly transcribed (Ventura et al. 2006). A similar transcriptional pattern wad identified for *L. gasseri* LgaI and *L. salivarius* Sal1, Sal2, Sal3 and Sal4 (Ventura et al. 2006). The presence of mRNA in the presumed phage repressor and superinfection exclusion genes and the lack of expression of the cro-like gene have been reported (Ventura et al. 2006).

3.10 Plasmid

A plasmid is a type of extrachromosomal genetic material that is often found in LAB strains (Table 3.5). As a member of various mobile genetic elements, it contributes to genome plasticity, host competitiveness and environment adaptation. Important plasmid-encoded genes associated with lactose/galactose utilisation, proteolysis, oligopeptide transport, bacteriophage resistance, citrate utilisation, EPS production, bacteriocin production, antibiotic resistance, heavy metal resistance and stress response were discovered in LAB species several decades ago (Schroeter and Klaenhammer 2009). It is noteworthy that most of these functions can be mapped in detail in *Lactoc. lactis* strains and some lactobacilli and pediococci but are not frequently present in *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and intestinal lactobacilli (Siezen et al. 2005; Davidson et al. 1996). It has been reported that *Lactoc. lactis* strains have been found to harbour several plasmids, which range in size from 2 to 80 kb (Davidson et al. 1996).

Table 3.5 General information of some LAB plasmids with interesting properties

Plasmid name	Host strain	Size	Function	Reference
pNZ4000	*Lactococcus lactis* ssp. *cremoris* NIZO B40	42.2 kb	Exopolysaccharide biosynthesis	van Kranenburg et al. (2000)
pMRC01	*Lactococcus lactis* ssp. *lactis* DPC3147	60.2 kb	Bacteriocin-producing	Dougherty et al. (1998)
pSK112	*Streptococcus cremoris* SK11	34 MDa	Bacteriophage resistance	de Vosetg al. (1984)
pCI301	*Lactococcus lactis* ssp. *lactis* UC317	75 kb	Proteinase	Law et al. (1992)
pSK11L	*Lactococcus lactis* ssp. *cremoris* SK11	47.2 kb	Oligopeptide transport and lactose utilization	Siezen et al. (2005)
pMP118	*Lactobacillus salivarius* UCC118	242 kb	Amino acid metabolism, carbohydrate utilization, bacteriocin-producing and bile salt hydrolase	Fang et al. (2008)
pOENI-1	*Oenococcus oeni* C9	18.3 kb	Wine adaptation	Favier et al. (2012)
pOENI-1v2	*Oenococcus oeni* S11	21.9 kb	Wine adaptation	Favier et al. (2012)
pCD01	*Lactobacillus paracasei* NFBC338	19.9 kb	Multidrug resistance	
pLgLA39	*Lactobacillus gasseri* LA39	33.3 kb	Gassericin A production/immunity	Ito et al. (2009)
pMD5057	*Lactobacillus plantarum* 5057	10.9 kb	Tetracycline resistance	Danielsen (2002)
pLR581	*Lactobacillus reuteri* ATCC 55730	–	Tetracycline resistance	Rosander et al. (2008)
pLR585		–	Lincomycin resistance	Rosander et al. (2008)
pLH1	*Lactobacillus helveticus* ATCC15009	19.4 kb	Relevant to milk fermentation	Thompson et al. (1999)
pLME300	*Lactobacillus fermentum* ROT1	19.4 kb	Erythromycin resistant and dalfopristin-resistance	Gfeller et al. (2003)
pLB925A04	*Lactobacillus brevis* 925A	65.0 kb	Brevicin 925A production	Wada et al. (2009)
plca36	*Lactobacillus casei* Zhang	36.5 kb	Toxin-antitoxin system	Zhang et al. (2008)
pKB290-1	*Lactobacillus brevis* KB290	42.4 kb	Cell wall biogenesis	Fukao et al. (2013)
pKB290-2		35.4 kb	Acid and bile resistance	Fukao et al. (2013)
pKB290-3		35.3 kb	Fatty acid synthesis	Fukao et al. (2013)
pKB290-5		17.9 kb	Tolerance to oxidative stress	Fukao et al. (2013)

For example, *Lactoc. lactis* SK11, a phage-resistant strain used in cheese making, contains four plasmids and was shown to have a substantial number of 'adaptation' genes (Siezen et al. 2005).

It is likely that plasmids harbour genes that may prove essential for survival under extreme conditions. The putative functions of proteins encoded by 34 *Lactobacillus* plasmids were re-classified with Clusters of Orthologous Groups by Zhang et al. (2008). Of the 576 genes analysed, approximately 250 were hypothetical genes, and putative functions could be assigned to only 44 of these. Genes encoding carbohydrate- and amino-acid-transport- and metabolism-related proteins composed the largest groups, consistent with the fermentability of *Lactobacillus* strains and the fact that *Lactobacillus* strains lost at least part of their ability to synthesise most amino acids as the result of adaptation to protein-rich environments. Seven cell-defence-related genes were identified in *L. acidophilus*, *L. casei*, *L. salivarius*, *L. fermentum*, and *L. sakei*, and these mainly encode ABC-type bacteriocin/lantibiotic exporters and type I/II restriction-modification systems, which promote the competition ability of LAB in nutrition-limited and other harsh conditions.

One of the most important plasmids discovered in LAB is the 242-kb plasmid pMP118 harboured by *L. salivarius* UCC118, which is larger than the reported 110-kb plasmid in *L. acidophilus* and the 150-kb plasmid in *L. gasseri* (Claesson et al. 2006). Contingency amino-acid-metabolism-related genes and carbohydrate-utilisation-related genes, including two genes for the completion of the pentose phosphate pathway and genes for the Abp118 bacteriocin and a bile salt hydrolase that is potentially relevant for probiotic properties, were identified in the megaplasmid (Claesson et al. 2006). This finding promoted the detection of the occurrence of megaplasmids in LAB and their functions. As described by Li et al. (2007), megaplasmids with a replication origin similar to that of pMP118 are widespread in 33 strains of *L. salivarius*. The extension of this investigation to other species revealed that megaplasmids with sizes ranging from 120 to 490 kb are found in *L. hamsteri*, *L. intestinalis*, *L. kalixensis*, *L. ingluviei*, *L. acidophilus* and *L. equi* strains (Li et al. 2007).

More recently, two newly announced genome projects revealed the largest plasmid complement within LAB. The draft genome of *L. plantarum* strain 16 contains more than eight plasmids, whereas nine plasmids were found in the completed genome of *L. brevis* KB290 (Crowley et al. 2013; Fukao et al. 2013). In *L. brevis* KB290, all of the plasmids had putative replication systems, including *repABC* genes for essential plasmid replication and stability, which is likely to explain the co-existence of multiple plasmids. Consistent with the presence of genes encoding EPS biosynthesis in the pKB290-1 plasmid, the plasmid appeared to be essential for the strains' GI tract tolerance and tendency to aggregate during plasmid-curing experiments. Genes encoding putative multidrug resistance transporters, enzymes associated with polyketide biosynthesis, cycling thioester proteins, and enzymes associated with the condensation of dipeptides with fatty acids and cysteine-dipeptide synthesis were found to be harboured by other plasmids and were found to be associated with the stress response (Fukao et al. 2013).

As mobile elements, plasmids in LAB occasionally confer properties of technological interest, such as improvements in the growth and behaviour of their host cells, which is particularly important in food production. Notably, *O. oeni*, which is the main LAB encountered in wine, contains plasmids associated with industrial starters and indigenous strains performing spontaneous malolactic fermentation that likely contribute to the technological performance of the strains in wine (Favier et al. 2012). Favier et al. (2012) indicated that two closely related plasmids, denoted pOENI-1 (18.3 kb) and pOENI-1v2 (21.9 kb), harbour two genes that are likely involved in wine adaptation; these encode a predicted sulphite exporter (*tauE*) and an NADH:flavin oxidoreductase of the old yellow enzyme family (*oye*). This hypothesis was demonstrated by an analysis of 95 wines at different phases of winemaking, which showed that strains carrying plasmids with the *tauE* and *oye* genes are predominant during spontaneous malolactic fermentation.

3.11 Summary and Perspectives

At present, the available genome sequences from different LAB species have greatly improved our understanding of their biology in nearly all aspects, particularly with respect to their industry-relevant important properties, e.g. metabolic and biosynthetic capabilities, cell surface factors, EPS biosynthetic pathway and bacteriocin secretion mechanisms. Benefiting from the knowledge of this useful information, the food industry, particularly the production of sourdough, vegetables, wine, dairy and meat products, has made significant progress in the past decades. However, a ‘dark’ area of biodiversity and evolution of LAB still exists due to a bias associated with the species or strains selected for sequencing. In the next few years, due to the rapid advances in sequencing technology, we will have unprecedented opportunities to expand our knowledge of LAB genetics. The increasing availability of genome data and the tools for genomics research, transcriptomics, proteomics, metabolomics and data modelling, will no doubt guide us to a new ‘omics’ era and facilitate the use of LAB in further industry applications.

References

Adams MR. Safety of industrial lactic acid bacteria. J Biotechnol. 1999;68(2–3):171–8.

Ai L, Chen C, Zhou F, Wang L, Zhang H, Chen W et al. Complete genome sequence of the probiotic strain *Lactobacillus casei* BD-II. J Bacteriol. 2011;193(12):3160–1.

Ainsworth S, Zomer A, de Jager V, Bottacini F, van Hijum SA, Mahony J et al. Complete genome of *Lactococcus lactis* subsp. *cremoris* UC509.9, host for a model *Lactococcal* P335 bacteriophage. Genome Announc. 2013;1(1):e00119–12.

Alcantara C, Revilla-Guarinos A, Zuniga M. Influence of two-component signal transduction systems of *Lactobacillus casei* BL23 on tolerance to stress conditions. Appl Environ Microbiol. 2010;77(4):1516–9.

Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O et al. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA. 2005;102(11):3906–12.

Aureli P, Capurso L, Castellazzi AM, Clerici M, Giovannini M, Morelli L et al. Probiotics and health: an evidence-based review. Pharmacol Res. 2011;63(5):366–76.

Azcarate-Peril MA, Altermann E, Goh YJ, Tallon R, Sanozky-Dawes RB, Pfeiler EA et al. Analysis of the genome sequence of *Lactobacillus gasseri* ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism. Appl Environ Microbiol. 2008;74(15):4610–25.

Azcarate-Peril MA, McAuliffe O, Altermann E, Lick S, Russell WM, Klaenhammer TR. Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. Appl Environ Microbiol. 2005;71(10):5794–804.

Barrangou R, Altermann E, Hutkins R, Cano R, Klaenhammer TR. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. Proc Natl Acad Sci USA. 2003;100(15):8957–62.

Ben Amor K, Vaughan EE, de Vos WM Advanced molecular tools for the identification of lactic acid bacteria. J Nutr. 2007;137(3 Suppl 2):741S–7S. doi:137/3/741S.

Berger B, Pridmore RD, Barretto C, Delmas-Julien F, Schreiber K, Arigoni F et al. Similarity and differences in the *Lactobacillus acidophilus* group identified by polyphasic analysis and comparative genomics. J Bacteriol. 2007;189(4):1311–21.

Bergonzelli GE, Granato D, Pridmore RD, Marvin-Guy LF, Donnicola D, Corthesy-Theulaz IE. GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. Infect Immun. 2006;74(1):425–34.

Boekhorst J, Helmer Q, Kleerebezem M, Siezen RJ. Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. Microbiology 2006;152(Pt 1):273–80.

Boekhorst J, Siezen RJ, Zwahlen MC, Vilanova D, Pridmore RD, Mercenier A et al. The complete genomes of *Lactobacillus plantarum* and *Lactobacillus johnsonii* reveal extensive differences in chromosome organization and gene content. Microbiology 2004;150(Pt 11):3601–11.

Boekhorst J, Wels M, Kleerebezem M, Siezen RJ. The predicted secretome of *Lactobacillus plantarum* WCFS1 sheds light on interactions with its environment. Microbiology 2006;152(Pt 11):3175–83.

Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, Kulakauskas S et al. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol. 2004;22(12):1554–8.

Bolotin A, Mauger S, Malarme K, Ehrlich SD, Sorokin A. Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. Antonie Van Leeuwenhoek. 1999;76(1–4):27–76.

Bongaerts GP, Severijnen RS. The beneficial, antimicrobial effect of probiotics. Med Hypotheses. 2001;56(2):174–7.

Borneman AR, McCarthy JM, Chambers PJ, Bartowsky EJ. Comparative analysis of the *Oenococcus oeni* pan genome reveals genetic diversity in industrially-relevant pathways. BMC Genomics. 2012;13:373. doi:1471-2164-13-373.

Broadbent JR, Neeno-Eckwall EC, Stahl B, Tandee K, Cai H, Morovic W et al. Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation. BMC Genomics. 2012;13:533.

Buck BL, Altermann E, Svingerud T, Klaenhammer TR. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol. 2005;71(12):8344–51.

Burgess C, O'Connell-Motherway M, Sybesma W, Hugenholtz J, van Sinderen D. Riboflavin production in *Lactococcus lactis*: potential for in situ production of vitamin-enriched foods. Appl Environ Microbiol. 2004;70(10):5769–77.

Cai H, Thompson R, Budinich MF, Broadbent JR, Steele JL. Genome sequence and comparative genome analysis of *Lactobacillus casei*: insights into their niche-associated evolution. Genome Biol Evol. 2009;1:239–57.

Callanan M, Kaleta P, O'Callaghan J, O'Sullivan O, Jordan K, McAuliffe O et al. Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. J Bacteriol. 2008;190(2):727–35.

Castaldo C, Vastano V, Siciliano RA, Candela M, Vici M, Muscariello L et al. Surface displaced alfa-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. Microb Cell Fact. 2009;8:14.

Chaillou S, Champomier-Verges MC, Cornet M, Crutz-Le Coq AM, Dudez AM, Martin V et al. The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23 K. Nat Biotechnol. 2005;23(12):1527–33.

Chang C, Stewart RC. The two-component system. Regulation of diverse signaling pathways in prokaryotes and eukaryotes. Plant Physiol. 1998;117(3):723–31.

Chen C, Ai L, Zhou F, Wang L, Zhang H, Chen W et al. Complete genome sequence of the probiotic bacterium *Lactobacillus casei* LC2W. J Bacteriol. 2011;193(13):3419–20.

Chopin MC, Chopin A, Rouault A, Galleron N. Insertion and amplification of foreign genes in the *Lactococcus lactis* subsp. *lactis* chromosome. Appl Environ Microbiol. 1989;55(7):1769–74.

Chopin A, Bolotin A, Sorokin A, Ehrlich SD, Chopin M. Analysis of six prophages in *Lactococcus lactis* IL1403: different genetic structure of temperate and virulent phage populations. Nucleic Acids Res. 2001;29(3):644–51.

Claesson MJ, Li Y, Leahy S, Canchaya C, van Pijkeren JP, Cerdeno-Tarraga AM et al. Multireplicon genome architecture of *Lactobacillus salivarius*. Proc Natl Acad Sci USA. 2006;103(17):6718–23.

Claesson MJ, van Sinderen D, O'Toole PW. *Lactobacillus* phylogenomics—towards a reclassification of the genus. Int J Syst Evol Microbiol. 2008;58(Pt 12):2945–54.

Crowley S, Bottacini F, Mahony J, van Sinderen D. Complete genome sequence of *Lactobacillus plantarum* strain 16, a broad-spectrum antifungal-producing lactic acid bacterium. Genome Announc. 2013;1(4):e00533–13.

Curry A. Archaeology: the milk revolution. Nature 2013;500(7460):20–22.

Dal Bello F, Walter J, Hammes WP, Hertel C. Increased complexity of the species composition of lactic acid bacteria in human feces revealed by alternative incubation condition. Microb Ecol. 2003;45(4):455–63.

Danielsen M. Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. Plasmid 2002;48(2):98–103.

Davidson BE, Kordias N, Dobos M, Hillier AJ. Genomic organization of lactic acid bacteria. Antonie Van Leeuwenhoek. 1996;70(2–4):161–83.

de Leeuw E, Li X, Lu W. Binding characteristics of the *Lactobacillus brevis* ATCC 8287 surface layer to extracellular matrix proteins. FEMS Microbiol Lett. 2006;260(2):210–15.

de Vos WM, Underwood HM, Davies FL. Plasmid encoded bacteriophage resistance in *Streptococcus cremoris* SK11. FEMS Microbiol Lett. 1984;23(2–3):175–8.

Delorme C, Bartholini C, Luraschi M, Pons N, Loux V, Almeida M et al. Complete genome sequence of the pigmented *Streptococcus thermophilus* strain JIM8232. J Bacteriol. 2011;193(19):5581–2.

Desiere F, Lucchini S, Canchaya C, Ventura M, Brussow H. Comparative genomics of phages and prophages in lactic acid bacteria. Antonie Van Leeuwenhoek. 2002;82(1–4):73–91.

Diep DB, Godager L, Brede D, Nes IF. Data mining and characterization of a novel pediocin-like bacteriocin system from the genome of *Pediococcus pentosaceus* ATCC 25745. Microbiology 2006;152(Pt 6):1649–59.

Dobson AE, Sanozky-Dawes RB, Klaenhammer TR. Identification of an operon and inducing peptide involved in the production of lactacin B by *Lactobacillus acidophilus*. J Appl Microbiol. 2007;103(5):1766–78.

Dougherty BA, Hill C, Weidman JF, Richardson DR, Venter JC, Ross RP. Sequence and analysis of the 60 kb conjugative, bacteriocin-producing plasmid pMRC01 from *Lactococcus lactis* DPC3147. Mol Microbiol. 1998;29(4):1029–38.

Falagas ME, Rafailidis PI, Makris GC. Bacterial interference for the prevention and treatment of infections. Int J Antimicrob Agents. 2008;31(6):518–22.

Fang F, Flynn S, Li Y, Claesson MJ, van Pijkeren JP, Collins JK et al. Characterization of endogenous plasmids from *Lactobacillus salivarius* UCC118. Appl Environ Microbiol. 2008;74(10):3216–28.

Favier M, Bilhere E, Lonvaud-Funel A, Moine V, Lucas PM. Identification of pOENI-1 and related plasmids in *Oenococcus oeni* strains performing the malolactic fermentation in wine. PLoS One. 2012;7(11):e49082.

Felis GE, Dellaglio F. Taxonomy of Lactobacilli and Bifidobacteria. Curr Issues Intest Microbiol. 2007;8(2):44–61.

Flynn S, van Sinderen D, Thornton GM, Holo H, Nes IF, Collins JK. Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118. Microbiology. 2002;148(Pt 4):973–84.

Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, et al. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World J Gastroenterol. 2007;13(2):236–43.

Forde BM, Neville BA, O'Donnell MM, Riboulet-Bisson E, Claesson MJ, Coghlan A et al. Genome sequences and comparative genomics of two *Lactobacillus ruminis* strains from the bovine and human intestinal tracts. Microb Cell Fact. 2011;10(Suppl 1):S13.

Forde B, Neville B, Donnell M, Riboulet-Bisson E, Claesson M, Coghlan A, et al. Genome sequences and comparative genomics of two *Lactobacillus ruminis* strains from the bovine and human intestinal tracts. Microb Cell Fact. 2011;10(suppl):S13.

Fukao M, Oshima K, Morita H, Toh H, Suda W, Kim SW et al. Genomic analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. PLoS One. 2013;8(3):e60521.

Gao Y, Lu Y, Teng KL, Chen ML, Zheng HJ, Zhu YQ et al. Complete genome sequence of *Lactococcus lactis* subsp. *lactis* CV56, a probiotic strain isolated from the vaginas of healthy women. J Bacteriol. 2011;193(11):2886–7.

German B, Schiffrin EJ, Reniero R, Mollet B, Pfeifer A, Neeser JR. The development of functional foods: lessons from the gut. Trends Biotechnol. 1999;17(12):492–9.

Gfeller KY, Roth M, Meile L, Teuber M. Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. Plasmid. 2003;50(3):190–201.

Gilliland SE. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol Rev. 1990;7(1–2):175–88.

Goh YJ, Klaenhammer TR. Genomic features of *Lactobacillus* species. Front Biosci (Landmark Ed). 2009;14:1362–86.

Goh YJ, Zhang C, Benson AK, Schlegel V, Lee JH, Hutkins RW. Identification of a putative operon involved in fructooligosaccharide utilization by *Lactobacillus paracasei*. Appl Environ Microbiol. 2006;72(12):7518–30.

Gorbach SL. Lactic acid bacteria and human health. Ann Med. 1990;22(1):37–41.

Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Corthesy-Theulaz IE. Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. Infect Immun. 2004;72(4):2160–9.

Guinane CM, Kent RM, Norberg S, Hill C, Fitzgerald GF, Stanton C, et al. Host specific diversity in *Lactobacillus johnsonii* as evidenced by a major chromosomal inversion and phage resistance mechanisms. PLoS ONE. 2011;6(4):e18740.

Hao P, Zheng H, Yu Y, Ding G, Gu W, Chen S, et al. Complete sequencing and pan-genomic analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* reveal its genetic basis for industrial yogurt production. PLoS ONE. 2011;6(1):e15964.

Hassan AN. ADSA foundation scholar award: possibilities and challenges of exopolysaccharide-producing lactic cultures in dairy foods. J Dairy Sci. 2008;91(4):1282–98.

Hendrix RW, Smith MC, Burns RN, Ford ME, Hatfull GF. Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. Proc Natl Acad Sci USA. 1999;96(5):2192–7.

Hochwind K, Weinmaier T, Schmid M, van Hemert S, Hartmann A, Rattei T et al. Draft genome sequence of *Lactobacillus casei* W56. J Bacteriol. 2012;194(23):6638.

Hols P, Hancy F, Fontaine L, Grossiord B, Prozzi D, Leblond-Bourget N et al. New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. FEMS Microbiol Rev. 2005;29(3):435–63.

Hosono A, Lee J, Ametani A, Natsume M, Hirayama M, Adachi T, et al. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from Bifidobacterium adolescentis M101-4. Biosci Biotechnol Biochem. 1997;61(2):312–6.

Hugenholtz J, Kleerebezem M. Metabolic engineering of lactic acid bacteria: overview of the approaches and results of pathway rerouting involved in food fermentations. Curr Opin Biotechnol. 1999;10(5):492–7.

Hugenholtz J, Sybesma W, Groot MN, Wisselink W, Ladero V, Burgess K, et al. Metabolic engineering of lactic acid bacteria for the production of nutraceuticals. Antonie Van Leeuwenhoek. 2002;82(1–4):217–35.

Ito Y, Kawai Y, Arakawa K, Honme Y, Sasaki T, Saito T. Conjugative plasmid from *Lactobacillus gasseri* LA39 that carries genes for production of and immunity to the circular bacteriocin gassericin A. Appl Environ Microbiol. 2009;75(19):6340–51.

Jamal Z, Miot-Sertier C, Thibau F, Dutilh L, Lonvaud-Funel A, Ballestra P et al. Distribution and functions of phosphotransferase system genes in the genome of the lactic acid bacterium *Oenococcus oeni*. Appl Environ Microbiol. 2013;79(11):3371–9.

Jimenez E, Langa S, Martin V, Arroyo R, Martin R, Fernandez L et al. Complete genome sequence of *Lactobacillus fermentum* CECT 5716, a probiotic strain isolated from human milk. J Bacteriol. 2010;192(18):4800.

Johansson P, Paulin L, Sade E, Salovuori N, Alatalo ER, Bjorkroth KJ et al. Genome sequence of a food spoilage lactic acid bacterium, *Leuconostoc gasicomitatum* LMG 18811T, in association with specific spoilage reactions. Appl Environ Microbiol. 2011;77(13):4344–51.

Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM. Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. Cell Microbiol. 2007;9(2):356–67.

Jolly L, Stingele F. Molecular organization and functionality of exopolysaccharide gene clusters in lactic acid bacteria. Int Dairy J. 2001;11(9):733–45.

Jung JY, Lee SH, Jeon CO. Complete genome sequence of *Leuconostoc carnosum* strain JB16, isolated from kimchi. J Bacteriol. 2012a;194(23):6672–73.

Jung JY, Lee SH, Jeon CO. Complete genome sequence of Leuconostoc gelidum strain JB7, isolated from kimchi. J Bacteriol. 2012b;194(23):6665.

Jung JY, Lee SH, Jeon CO. Complete genome sequence of *Leuconostoc mesenteroides* subsp. *mesenteroides* strain J18, isolated from kimchi. J Bacteriol. 2012c;194(3):730–1.

Kaleta P, O'Callaghan J, Fitzgerald GF, Beresford TP, Ross RP. Crucial role for insertion sequence elements in *Lactobacillus helveticus* evolution as revealed by interstrain genomic comparison. Appl Environ Microbiol. 2009;76(1):212–20.

Kandler O. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek. 1983;49(3):209–24.

Kang X, Ling N, Sun G, Zhou Q, Zhang L, Sheng Q. Complete genome sequence of *Streptococcus thermophilus* strain MN-ZLW-002. J Bacteriol. 2012;194(16):4428–29.

Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P et al. Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein. Proc Natl Acad Sci USA. 2009;106(40):17193–8.

Kato H, Shiwa Y, Oshima K, Machii M, Araya-Kojima T, Zendo T et al. Complete genome sequence of *Lactococcus lactis* IO-1, a lactic acid bacterium that utilizes xylose and produces high levels of L-lactic acid. J Bacteriol. 2012;194(8):2102–3.

Kergourlay G, Messaoudi S, Dousset X, Prevost H. Genome sequence of *Lactobacillus salivarius* SMXD51, a potential probiotic strain isolated from chicken cecum, showing anti-campylobacter activity. J Bacteriol. 2012;194(11):3008–9.

Kim JF, Jeong H, Lee JS, Choi SH, Ha M, Hur CG et al. Complete genome sequence of *Leuconostoc citreum* KM20. J Bacteriol. 2008;190(8):3093–4.

Klaenhammer T, Altermann E, Arigoni F, Bolotin A, Breidt F, Broadbent J, et al. Discovering lactic acid bacteria by genomics. Antonie Van Leeuwenhoek. 2002;82(1–4):29–58.

Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R et al. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci USA. 2003;100(4):1990–5.

Klein G, Pack A, Bonaparte C, Reuter G. Taxonomy and physiology of probiotic lactic acid bacteria. Int J Food Microbiol. 1998;41(2):103–25.

Kok J. Genetics of the proteolytic system of lactic acid bacteria. FEMS Microbiol Rev. 1990;7(1–2):15–42.

Landete JM, Garcia-Haro L, Blasco A, Manzanares P, Berbegal C, Monedero V et al. Requirement of the *Lactobacillus casei* MaeKR two-component system for L-malic acid utilization via a malic enzyme pathway. Appl Environ Microbiol. 2009;76(1):84–95.

Law J, Vos P, Hayes F, Daly C, de Vos WM, Fitzgerald G. Cloning and partial sequencing of the proteinase gene complex from *Lactococcus lactis* subsp. *lactis* UC317. J Gen Microbiol. 1992;138(4):709–18.

Lee SH, Jung JY, Jeon CO. Complete genome sequence of *Weissella koreensis* KACC 15510, isolated from kimchi. J Bacteriol. 2011;193(19):5534.

Li X, Gu Q, Lou X, Zhang X, Song D, Shen L et al. Complete genome sequence of the probiotic *Lactobacillus plantarum* strain ZJ316. Genome Announc. 2013;1(2):e00094–13.

Li Y, Canchaya C, Fang F, Raftis E, Ryan KA, van Pijkeren JP et al. Distribution of megaplasmids in *Lactobacillus salivarius* and other lactobacilli. J Bacteriol. 2007;189(17):6128–39.

Linares DM, Kok J, Poolman B. Genome sequences of *Lactococcus lactis* MG1363 (revised) and NZ9000 and comparative physiological studies. J Bacteriol. 2010;192(21):5806–12.

Liu M, Bayjanov JR, Renckens B, Nauta A, Siezen RJ. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics. 2010;11:36.

Lunde M, Blatny JM, Lillehaug D, Aastveit AH, Nes IF. Use of real-time quantitative PCR for the analysis of phiLC3 prophage stability in lactococci. Appl Environ Microbiol. 2003;69(1):41–8.

Macklaim JM, Gloor GB, Anukam KC, Cribby S, Reid G. At the crossroads of vaginal health and disease, the genome sequence of *Lactobacillus iners* AB-1. Proc Natl Acad Sci USA. 2010;108(Suppl 1):4688–95.

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E et al. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci USA. 2006;103(42):15611–6.

Makarova KS, Koonin EV. Evolutionary genomics of lactic acid bacteria. J Bacteriol. 2007;189(4):1199–208.

Martinez-Cuesta Mdel C, Pelaez C, Requena T. Methionine metabolism: major pathways and enzymes involved and strategies for control and diversification of volatile sulfur compounds in cheese. Crit Rev Food Sci Nutr. 2013;53(4):366–85.

Mayo B. The proteolytic system of lactic acid bacteria. Microbiologia. 1993;9(2):90–106.

Maze A, Boel G, Zuniga M, Bourand A, Loux V, Yebra MJ et al. Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. J Bacteriol. 2010;192(10):2647–8.

Mills DA, Rawsthorne H, Parker C, Tamir D, Makarova K. Genomic analysis of *Oenococcus oeni* PSU-1 and its relevance to winemaking. FEMS Microbiol Rev. 2005;29(3):465–75.
Miyoshi Y, Okada S, Uchimura T, Satoh E. A mucus adhesion promoting protein, MapA, mediates the adhesion of *Lactobacillus reuteri* to Caco-2 human intestinal epithelial cells. Biosci Biotechnol Biochem. 2006;70(7):1622–8.
Moon YJ, Soh JR, Yu JJ, Sohn HS, Cha YS, Oh SH. Intracellular lipid accumulation inhibitory effect of *Weissella koreensis* OK1-6 isolated from Kimchi on differentiating adipocyte. J Appl Microbiol. 2012;113(3):652–8.
Morel-Deville F, Fauvel F, Morel P. Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei*. Microbiology. 1998;144(Pt 10):2873–83.
Morita H, Toh H, Fukuda S, Horikawa H, Oshima K, Suzuki T et al. Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. DNA Res. 2008;15(3):151–61.
Morita H, Toh H, Oshima K, Murakami M, Taylor TD, Igimi S et al. Complete genome sequence of the probiotic *Lactobacillus rhamnosus* ATCC 53103. J Bacteriol. 2009;191(24):7630–1.
Munoz-Provencio D, Perez-Martinez G, Monedero V. Characterization of a fibronectin-binding protein from *Lactobacillus casei* BL23. J Appl Microbiol. 2009;108(3):1050–9.
Navarre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev. 1999;63(1):174–229.
Nelson KE. The future of microbial genomics. Environ Microbiol. 2003;5(12):1223–5.
Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek. 1996;70(2–4):113–28.
Nyquist OL, McLeod A, Brede DA, Snipen L, Aakra A, Nes IF. Comparative genomics of *Lactobacillus sakei* with emphasis on strains from meat. Mol Genet Genomics. 2011;285(4):297–311.
O'Connell-Motherway M, van Sinderen D, Morel-Deville F, Fitzgerald GF, Ehrlich SD, Morel P. Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363. Microbiology. 2000;146(Pt 4):935–47.
Oh HM, Cho YJ, Kim BK, Roe JH, Kang SO, Nahm BH et al. Complete genome sequence analysis of *Leuconostoc kimchii* IMSNU 11154. J Bacteriol. 2010;192(14):3844–5.
Oh S, Roh H, Ko HJ, Kim S, Kim KH, Lee SE et al. Complete genome sequencing of *Lactobacillus acidophilus* 30SC, isolated from swine intestine. J Bacteriol. 2011;193(11):2882–3.
Pal K, Szen O, Kiss A, Naar Z. Comparison and evaluation of molecular methods used for identification and discrimination of lactic acid bacteria. J Sci Food Agric. 2012;92(9):1931–6.
Pastink MI, Teusink B, Hols P, Visser S, de Vos WM, Hugenholtz J. Genome-scale model of *Streptococcus thermophilus* LMG18311 for metabolic comparison of lactic acid bacteria. Appl Environ Microbiol. 2009;75(11):3627–33.
Peant B, LaPointe G, Gilbert C, Atlan D, Ward P, Roy D. Comparative analysis of the exopolysaccharide biosynthesis gene clusters from four strains of *Lactobacillus rhamnosus*. Microbiology 2005;151(Pt 6):1839–51.
Pfeiler EA, Azcarate-Peril MA, Klaenhammer TR. Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. J Bacteriol. 2007;189(13):4624–34.
Pittet V, Ewen E, Bushell BR, Ziola B. Genome sequence of *Lactobacillus rhamnosus* ATCC 8530. J Bacteriol. 2012;194(3):726.
Postma PW, Lengeler JW, Jacobson GR. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol Rev. 1993;57(3):543–94.
Pretzer G, Snel J, Molenaar D, Wiersma A, Bron PA, Lambert J et al. Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. J Bacteriol. 2005;187(17):6128–36.

Pridmore RD, Berger B, Desiere F, Vilanova D, Barretto C, Pittet AC et al. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc Natl Acad Sci USA. 2004;101(8):2512–7.

Rasmussen TB, Danielsen M, Valina O, Garrigues C, Johansen E, Pedersen MB. *Streptococcus thermophilus* core genome: comparative genome hybridization study of 47 strains. Appl Environ Microbiol. 2008;74(15):4703–10.

Reuter G. Elective and selective media for lactic acid bacteria. Int J Food Microbiol. 1985;2(1–2):55–68.

Revilla-Guarinos A, Gebhard S, Alcantara C, Staron A, Mascher T, Zuniga M. Characterization of a regulatory network of peptide antibiotic detoxification modules in *Lactobacillus casei* BL23. Appl Environ Microbiol. 2013;79(10):3160–70.

Roos S, Jonsson H. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology. 2002;148(Pt 2):433–42.

Rosander A, Connolly E, Roos S. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. Appl Environ Microbiol. 2008;74(19):6032–40.

Rossetti L, Giraffa G. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. J Microbiol Methods. 2005;63(2):135–144.

Ryan MP, Rea MC, Hill C, Ross RP. An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl Environ Microbiol. 1996;62(2):612–9.

Salvetti E, Torriani S, Felis GE. The genus *Lactobacillus*: a taxonomic update. Probiotics Antimicro Prot. 2012;4:217–26.

Saulnier DM, Molenaar D, de Vos WM, Gibson GR, Kolida S. Identification of prebiotic fructooligosaccharide metabolism in *Lactobacillus plantarum* WCFS1 through microarrays. Appl Environ Microbiol. 2007;73(6):1753–65.

Schroeter J, Klaenhammer T. Genomics of lactic acid bacteria. FEMS Microbiol Lett. 2009;292(1):1–6.

Shiby VK, Mishra HN. Fermented milks and milk products as functional foods—a review. Crit Rev Food Sci Nutr. 2013;53(5):482–96.

Siezen RJ, Bayjanov J, Renckens B, Wels M, van Hijum SA, Molenaar D et al. Complete genome sequence of *Lactococcus lactis* subsp. *lactis* KF147, a plant-associated lactic acid bacterium. J Bacteriol. 2010;192(10):2649–50.

Siezen RJ, Renckens B, van Swam I, Peters S, van Kranenburg R, Kleerebezem M et al. Complete sequences of four plasmids of *Lactococcus lactis* subsp. *cremoris* SK11 reveal extensive adaptation to the dairy environment. Appl Environ Microbiol. 2005;71(12):8371–82.

Siezen RJ, van Enckevort FH, Kleerebezem M, Teusink B. Genome data mining of lactic acid bacteria: the impact of bioinformatics. Curr Opin Biotechnol. 2004;15(2):105–15.

Siezen RJ, Bayjanov JR, Felis GE, van der Sijde MR, Starrenburg M, Molenaar D, et al. Genome-scale diversity and niche adaptation analysis of *Lactococcus lactis* by comparative genome hybridization using multi-strain arrays. Microb Biotechnol. 2011;4(3):383–402.

Stahl B, Barrangou R. Complete genome sequence of probiotic strain *Lactobacillus acidophilus* La-14. Genome Announc 2013;1(3):e00376–13.

Stiles ME. Biopreservation by lactic acid bacteria. Antonie Van Leeuwenhoek. 1996;70(2–4):331–45.

Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol. 1997;36:1–29.

Sturme MH, Nakayama J, Molenaar D, Murakami Y, Kunugi R, Fujii T et al. An agr-like two-component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J Bacteriol. 2005;187(15):5224–35.

Sun Z, Chen X, Wang J, Zhao W, Shao Y, Guo Z et al. Complete genome sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* strain ND02. J Bacteriol. 2011;193(13):3426–7.

Sun Z, Chen X, Wang J, Zhao W, Shao Y, Wu L et al. Complete genome sequence of *Streptococcus thermophilus* strain ND03. J Bacteriol. 2010;193(3):793–4.
Suyama M, Bork P. Evolution of prokaryotic gene order: genome rearrangements in closely related species. Trends Genet. 2001;17(1):10–13.
Tafti AG, Peighambardoust SH, Hesari J, Bahrami A, Bonab ES. Physico-chemical and functional properties of spray-dried sourdough in breadmaking. Food Sci Technol Int. 2013;19(3):271–8.
Taverniti V, Guglielmetti S. Health-promoting properties of *Lactobacillus helveticus*. Front Microbiol. 2012;3:392.
Thompson JK, Foley S, McConville KJ, Nicholson C, Collins MA, Pridmore RD. Complete sequence of plasmid pLH1 from *Lactobacillus helveticus* ATCC15009: analysis reveals the presence of regions homologous to other native plasmids from the host strain. Plasmid. 1999;42(3):221–35.
Tillier ER, Collins RA. Genome rearrangement by replication-directed translocation. Nat Genet. 2000;26(2):195–7.
Tompkins TA, Barreau G, Broadbent JR. Complete genome sequence of *Lactobacillus helveticus* R0052, a commercial probiotic strain. J Bacteriol. 2012;194(22):6349.
Vadeboncoeur C, Moineau S. The relevance of genetic analysis to dairy bacteria: building upon our heritage. Mirob Cell Fact. 2004;3:15.
van Kranenburg R, Kleerebezem M, de Vos WM. Nucleotide sequence analysis of the lactococcal EPS plasmid pNZ4000. Plasmid. 2000;43(2):130–6.
van Pijkeren JP, Canchaya C, Ryan KA, Li Y, Claesson MJ, Sheil B et al. Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. Appl Environ Microbiol. 2006;72(6):4143–53.
Vastano V, Salzillo M, Siciliano RA, Muscariello L, Sacco M, Marasco R. The E1 beta-subunit of pyruvate dehydrogenase is surface-expressed in *Lactobacillus plantarum* and binds fibronectin. Microbiol Res. 2014;169(3):121–7.
Ventura M, Canchaya C, Bernini V, Altermann E, Barrangou R, McGrath S et al. Comparative genomics and transcriptional analysis of prophages identified in the genomes of *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus casei*. Appl Environ Microbiol. 2006;72(5):3130–46.
Vogel RF, Pavlovic M, Ehrmann MA, Wiezer A, Liesegang H, Offschanka S et al. Genomic analysis reveals *Lactobacillus sanfranciscensis* as stable element in traditional sourdoughs. Microb Cell Fact. 2011;10(Suppl 1):S6.
von Ossowski I, Satokari R, Reunanen J, Lebeer S, De Keersmaecker SC, Vanderleyden J et al. Functional characterization of a mucus-specific LPXTG surface adhesin from probiotic *Lactobacillus rhamnosus* GG. Appl Environ Microbiol. 2011;77(13):4465–72.
Wada T, Noda M, Kashiwabara F, Jeon HJ, Shirakawa A, Yabu H et al. Characterization of four plasmids harboured in a *Lactobacillus brevis* strain encoding a novel bacteriocin, brevicin 925A, and construction of a shuttle vector for lactic acid bacteria and *Escherichia coli*. Microbiology 2009;155(Pt 5):1726–37.
Wang Y, Chen C, Ai L, Zhou F, Zhou Z, Wang L et al. Complete genome sequence of the probiotic *Lactobacillus plantarum* ST-III. J Bacteriol. 2010;193(1):313–4.
Wegmann U, O'Connell-Motherway M, Zomer A, Buist G, Shearman C, Canchaya C et al. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J Bacteriol. 2007;189(8):3256–70. doi:JB.01768-06.
Wegmann U, Overweg K, Horn N, Goesmann A, Narbad A, Gasson MJ et al. Complete genome sequence of *Lactobacillus johnsonii* FI9785, a competitive exclusion agent against pathogens in poultry. J Bacteriol. 2009;191(22):7142–3.
Welman AD, Maddox IS. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 2003;21(6):269–74.
Ya T, Zhang Q, Chu F, Merritt J, Bilige M, Sun T, et al. Immunological evaluation of *Lactobacillus casei* Zhang: a newly isolated strain from koumiss in Inner Mongolia. China BMC Immunol. 2008;9:68.

Yebra MJ, Zuniga M, Beaufils S, Perez-Martinez G, Deutscher J, Monedero V. Identification of a gene cluster enabling *Lactobacillus casei* BL23 to utilize myo-inositol. Appl Environ Microbiol. 2007;73(12):3850–8.

Zhang W, Sun Z, Wu R, Menghe, Zhang H. Comparative genome analysis of probiotic *Lactobacillus casei* Zhang. In: Genomics II: bacteria, viruses and metabolic pathways. 1st ed. Hongkong: iConcept Press Ltd; 2013. p. 276–96.

Zhang W, Yu D, Sun Z, Chen X, Bao Q, Meng H et al. Complete nucleotide sequence of plasmid plca36 isolated from *Lactobacillus casei* Zhang. Plasmid. 2008;60(2):131–5.

Zhang W, Yu D, Sun Z, Wu R, Chen X, Chen W et al. Complete genome sequence of *Lactobacillus casei* Zhang, a new probiotic strain isolated from traditional homemade koumiss in Inner Mongolia, China. J Bacteriol. 2010;192(19):5268–9.

Zhang ZY, Liu C, Zhu YZ, Wei YX, Tian F, Zhao GP et al. Safety assessment of *Lactobacillus plantarum* JDM1 based on the complete genome. Int J Food Microbiol. 2011;153(1–2):166–70.

Zhang W, Yu D, Sun Z, Chen W, Hu S, Meng H, et al. The comparative analysis of a prophage remnant Lcazh1 in relation to other *Lactobacillus* prophages, particularly Lp3. Int J Dairy Technol. 2010;63(3):413–7.

Zhang Y, Wang L, Zhang J, Li Y, He Q, Li H, et al. Probiotic *Lactobacillus casei* Zhang ameliorates high-fructose-induced impaired glucose tolerance in hyperinsulinemia rats. Eur J Nutr. 2014;53(1):221–32.

Zhao W, Chen Y, Sun Z, Wang J, Zhou Z, Sun T et al. Complete genome sequence of *Lactobacillus helveticus* H10. J Bacteriol. 2011;193(10):2666–7.

Zhu Y, Zhang Y, Li Y. Understanding the industrial application potential of lactic acid bacteria through genomics. Appl Microbiol Biotechnol. 2009;83(4):597–610.

Chapter 4
Proteomics of Lactic Acid Bacteria

Rina Wu and Jing Lu

Abstract In this chapter, the proteomics analysis of lactic acid bacteria (LAB) focused on the whole proteins of LAB and the response to environmental stress or growth condition was reviewed. Following the development of morden biological technology, more and more LAB genome has been finished. However, the efficient mining of these data requires the development of functional genomic tools, of which proteomic studies are one of the most important. LAB proteomic studies investigate the global protein expression profile of a LAB isolate rather than the behaviour of a single protein. The procedure includes protein separation and protein identification. Two-dimensional electrophoresis (2-DE), liquid chromatography (LC) and mass spectrometry (MS) are the major techniques used in LAB proteome studies. A comprehensive description of LAB proteins and analysis of their expression pattern under different environmental conditions would significantly increase our understanding of the metabolic mechanisms underlying the growth performance. Further, the LAB proteome information is necessary to achieve the highest quality and safest products in food fermentation.

Keywords Lactic acid bacteria · Proteomics · Stress response

The lactic acid bacteria (LAB) are a group of Gram-positive, nonmotile, nonspore forming firmicutes that ferment sugars, mainly to lactic acid, and have rod- or coccus-shaped cells (Coenye and Vandamme 2003). LAB are widespread in nature, including in the human digestive system, and are mainly beneficial contributing to human health (Salminen and Isolauri 2006). People now understand the beneficial function of LAB, thereby increasing their economic value; indeed the

R. Wu (✉)
Shenyang Agricultural University, Shenyang, Liaoning, China
e-mail: wrn6956@163.com

J. Lu
Chinese Academy of Agricultural Sciences, Institute of Agro-products Processing Science and Technology, Beijing, China
e-mail: ljangela@163.com

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_4,
© Springer Science+Business Media Dordrecht 2014

worldwide consumption of fermented food is increasing as it is an effective way to supplement LAB strains and improve health. To deliver health benefits LAB must survive in a variety of challenging niches in the intestine where they are exposed to gastric acid, bile salts, peristalsis and competition for space and nutrients (Bezkorovainy 2001). However, as starter cultures in industrial dairy fermentations, sourdough production, wine production, pickling and curing processes, LAB are adapted to environmental stresses such as extreme temperature and pH, high osmotic pressure and starvation (Marles-Wright and Lewis 2007).

As with other bacteria, the ability of the metabolic system of LAB to respond to stress is essential for tolerance to sudden changes in environmental conditions. Initial studies to understand stress responses focused on single genes or enzymes associated with the response. With the development of 'omic' technology, a huge amount of DNA data have become available that describe the genetic make-up of LAB. However, to explore the secrets of the stress response in detail, it is important to elucidate the protein molecules in cells that are directly responsible for the maintenance of correct cellular function (Haynes and Yates 2000). The expression patterns of whole cellular proteins are highly dynamic and complex (Curreem et al. 2012). Therefore, the study of the entire range of proteins expressed in a cell under environmental stress, known as the 'proteome', has become an area of great interest.

While genome sequences are a static view of an organism, proteomics introduce a quantitative and dynamic vision of how proteins vary throughout a cell's life. Many LAB genes have been sequenced and are available in the Genbank of the National Center for Biotechnology Information (NCBI). However, the efficient mining of these data requires the development of functional genomic tools, of which proteomic studies are one of the most important. In this chapter, we will describe the physiological and molecular mechanisms of environmental stress responses in LAB as elucidated using proteomic technology.

4.1 Introduction to Proteomics

The proteome is defined as the protein complement expressed by the genome of an organism or cell type (Anderson and Anderson 1996; Wasinger et al. 1995; Wilkins et al. 1996). Proteomic studies investigate the global protein expression profile rather than the behaviour of a single protein. Although the term was coined in 1994, the discipline itself had its origins in the electrophoretic separation techniques of the 1970s and 1980s.

Compared to genomic research, proteomic studies could be considered more challenging technically. For example, within a given human proteome, the number of proteins present may be as many as 2 million, whereas the genome is only about 2.91 Gbp (Hartl and Jones 2005). It is often inaccurate to depend solely on genomic information to infer the functions of proteins, or to rely purely on transcriptomic data to quantify protein expression levels (Curreem et al. 2012). The reason for this

is because there can be more than 200 different types of post-translational modification of proteins that are possible, including phosphorylation, glycosylation, acetylation, deamination, farnesylation, myristoylation, palmitoylation and proteolysis (Krishna and Wold 1993). From DNA sequences, that range of modifications cannot be predicted completely. Only a thorough study of the proteins themselves can elucidate their characteristics and functions. Hence, it is more accurate and informative, although often more technically demanding, to study proteins directly.

The goal of proteomics is to analyse the variation in proteomes of an organism that occur at different times and under different conditions, to highlight the changes (Graves and Haystead 2002). In brief, proteomics analyses the changes in structure and function of biological systems. For example, the protein content of a stress-influenced cell is often different from that of a normal cell. Some proteins in the stress-influenced cell may not be present in the normal cell, making these unique proteins good targets for controlling cell growth. To achieve this goal requires both purification and identification of the proteins and can be hindered by a multitude of biological and environmental factors (Van Wijk 2001).

4.1.1 Types of Proteomics

There are different subdivisions of proteomics: structural proteomics, expression proteomics and interaction proteomics. Expression proteomics analyse expression and differential expression of proteins. Protein expression of the entire proteome or of a subset of proteomes can be compared. Novel proteins (e.g. disease-specific biomarkers) can also be identified. Structural proteomics map the structure of protein complexes or the proteins present in a subcellular localisation or an organelle (Blackstock and Weir 1999). Interaction proteomics is the analysis of interactions between proteins and is used to characterise protein complexes and determine their function (Twyman 2003).

The aim of all types of proteomics is not only to identify all the proteins present but also to create a complete three-dimensional (3-D) map of proteins within a cell or an organism (Lau et al. 2003). To complete this map requires contributions from various disciplines such as biochemistry, molecular biology, biophysics and bioinformatics. However, it should be noted, that the proteome of a particular cell is in a dynamic state, and is likely to change at any moment in response to external stimuli. Thus, studying the proteome is similar to taking a snapshot of the global expression pattern at a particular time.

4.1.2 Proteomics Procedures and Technology

The procedure follows two steps: protein separation and protein identification. The technology used includes two-dimensional electrophoresis (2-DE) and liquid chromatography (LC) for protein separation, and mass spectrometry (MS) for

protein identification. MS can also be divided into two common methods: matrix-assisted laser detection of desorption/ionization (MALDI) and electrospray ionisation (ESI) (Strupat et al. 1994). Based on the characteristics of the protein sample loaded, 2-DE is always combined with MALDI-MS and LC is always combined with ESI-MS, forming two classical procedures for proteomic analysis.

4.1.2.1 2-DE and MALDI-MS

2-DE enables up to 10,000 proteins to be distinguished from each other on a gel. The method is based on two distinct physical and chemical features of proteins. First the proteins are separated according to their isoelectric point (p*I*) (Westermeier et al. 2008). After this separation in one direction, standard electrophoresis on a polyacrylamide gel (PAGE) is applied. Proteins then migrate in a second direction through the gel according to their size.

Sample preparation for 2-DE analysis is very important. The principle is to convert the sample into a suitable physiochemical state for first dimension isoelectric focusing (IEF) while preserving the natural charge and molecular weight (M_r) of the constituent proteins (Westermeier et al. 2008; Shaw and Riederer 2003). In general, deep-frozen cells or tissues are disrupted using various techniques such as grinding in a liquid nitrogen-cooled mortar, sonication, shearing-based methods or homogenisation (Görg et al. 2000). Proteins can then be solubilised by sonication in a lysis buffer (such as urea, thiourea, DTT, CHAP, carrier ampholytes, 1 % SDS or a cocktail of these buffers.) and then diluted by at least threefold in lysis buffer (Harder et al. 1999). The type of lysis buffer and its concentration depend on the type of sample. Trichloroacetic acid (TCA)/acetone extraction can also be included as a purification step.

Being Gram-positive bacteria, the cell walls of LAB are thicker than Gram-negative species. Hence, mechanical disruption is always required. It is recommended to degrade the nuclei of bacteria with RNAase and DNAase to avoid cross stripes on 2-DE maps (Wu et al. 2009a). Furthermore, the culture media used for LAB contain ions such as Mn^{2+} and Mg^{2+}. High ion concentrations can interfere with the focusing of 2-DE and so it is also recommended that bacteria are washed at least three times before evaluation.

After both phases of 2-DE, it is necessary to visualise the proteins using staining or labelling methods (chemical or radioactive). The resulting 'maps' of proteins can then be compared for differential expression between, for example, experimental and control samples which can then be linked with the mechanisms involved in the biological process. The identity of differentially expressed proteins is verified by 'cutting out' the area of the gel that is different and analysing the protein present by MS.

The role of MALDI-MS is to identify the protein and its modification by 'soft' ionisation methods. This results in the formation of ions without significant loss of sample integrity and enables accurate information on mass of proteins and peptides to be determined in their natural states (Graves and Haystead 2002). MALDI-MS

begins with sample preparation, sample ionisation and then analysis of mass: the protein is always first digested by trypsin into smaller peptides, and then mixed with matrix molecules. After irradiation using specific wavelengths of laser, the sample can be ionised by absorbing the energy from the matrix molecules. The mass to charge ratio (m/z) of the ions can then be measured by determining the time required for them to traverse the length of a time-of-flight (TOF) instrument. The spectrum of peptides present can then be identified by comparing them with theoretical spectra that have been calculated from protein sequences available in public databases, using peptide mass fingerprinting (PMF) and bioinformatics tools (James et al. 1993; Jensen et al. 1997; Mann et al. 1993; Yates et al. 1993; Pappin et al. 1993). Combining advanced MS techniques with database searching has played a crucial role in protein identification for proteomics studies (Singh and Nagaraj 2006).

In recent years, 2-DE has also been improved substantially with the introduction of difference gel electrophoresis (DIGE) technology. This technology uses fluorescent tagging of three reference protein samples, each with a different dye. These dyes are amine reactive and all have the same molecular mass to avoid changing the mass of each tagged sample by a different amount. The tagged proteins are mixed together and run on the 2-D gel at the same time as the unknown protein samples. After image acquisition by a fluorescent scanner at a number of different excitation wavelengths for each dye, the gel images are superimposed on each other to identify differences (Unlu et al. 1997). This technique minimises gel-to-gel variation and reduces the workload as the number of gels that have to be run are reduced. Compared with traditional colourimetric protein stains, fluorescent dyes have greater detection sensitivity and a good dynamic range. Therefore, 2D-DIGE is capable of quantifying small differences in protein levels, making it a popular approach for large-scale expression profiling in a range of biological systems. For proteomics studies of LAB, 2D-DIGE has become a standard comparative approach for the study of stress responses.

4.1.2.2 LC and ESI-MS

ESI-MS has always been one of the most important techniques in proteomics. Like MALDI-MS, it is also a ‘soft’ ionisation method. ESI is based on spraying a fine electrically generated mist of ions from the experimental and reference samples into the inlet of a mass spectrometer at atmospheric pressure (Guerrera and Kleiner 2005). In the ESI, the ion source (sample for evaluation) is placed in an ionisation chamber where intact molecular ions are produced. These ions are transferred to the mass analyser section of the equipment via several ion optics that focus the ion stream to maintain a stable trajectory. The mass analyser sorts and separates the ions according to their m/z value. The separated ions are then passed to detector systems to measure their concentration and the results are displayed on a mass spectrum chart (Banerjee and Mazumdar 2012). ESI is most frequently coupled to ion traps or quadruple time-of-flight (Q/TOF) instruments.

This technique ionises molecules directly from solution, so it can easily be interfaced with LC separation methods thereby opening a new analytical window for proteomics analysis (Joo and Kim 2005). Moreover, LC itself is able to supply the automatically purified peptide samples for ESI to improve detection accuracy. LC systems include LC, high-performance LC (HPLC) and reversed-phase LC (RP-LC) amongst others. HPLC can be used to remove buffers and salts and to separate the analyte from contaminants. LC-ESI technique has already been used in the direct identification of proteins in mixtures (Yates 1998), including the proteins involved in the stress responses of LAB, which we will illustrate in following sections. It can be combined with 1-D electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), or not. However, LC-ESI also has problems; large-sized samples of peptides are not easy to identify and short peptides may be lost. Overall, it is fair to say that there is still no single perfect method to analyse all the proteins in an organism or a cell.

4.1.2.3 Bioinformatics in Proteomics

The bioinformatics methods used in proteomics can be divided into 'statistical' and 'functional' approaches (Colinge and Bennett 2007). After 2-DE and protein visualisation by silver or coomassie blue staining (Rabilloud 1992; Candiano et al. 2004), gels are digitised in an image scanner for computer analysis, and subjected to specialist image analysis software according to the intensity (volume) of each spot. Usually, only significantly up or downregulated spots or present/absent spots are selected for analysis with MS. Statistical analysis includes MS spectrum detection and database searching.

Databases from NCBI, EMBL, SWISS-PROT, Uniport, etc. are available for protein identification. Several software packages are available for database matching including MASCOT at www.matrixscience.com (Perkins et al. 1999), ProFound at www.prowl.com (Zhang and Chait 2000) and Protein Prospector at www.prospector.ucsf.edu (Clauser et al. 1999). Moreover, databases of 2-DE maps have been established, e.g. SWISS-2D PAGE, the Rice Proteome Database, ECO-2DBASE of *E. coli* and YPD of yeast. LAB 2-DE images in SWISS-2D PAGE in particular have been used as references in LAB research. To analyse and predict the sequence, structure and p*I* of any protein, especially an unknown protein, a number of bioinformatic software systems are useful. The ExPASy package includes the AACompIdent toolkit that can search the amino acid composition of known proteins and compares them with the amino acid composition of an unknown protein to identify homology. The PROPSEARCH package also offers homology searches in relation to amino acid sequences but also in relation to molecular weight, two selected peptide components and other parameters; furthermore, it is free. Compute p*I*/MW, PeptideMass, TGREASE and SAPS software are available for the calculation of p*I* and molecular weight, enzyme restriction properties, hydrophobicity and the charge distribution of the proteins under investigation, respectively. Compute p*I*/MW and PeptideMass are both part

of the ExPASy toolkit. The Predict-Protein package predicts protein secondary structure. It first searches similar sequences in SWISS-PROT, uses these to construct a profile of multi-sequence comparisons using the MaxHom method and then predicts the structural features of the protein in question. The average accuracy rate for predictions made using this method is 72 %, but it can be as high as 90 %. Finally, Protein 3-D structural prediction is one of the most complicated bioimformatic techniques currently available because the process of protein folding is still not well understood. Software such as SWISS-MODEL and the CPH models can play a certain role in 3-D structural prediction.

Functional proteomics profiles are inherently complex as each of the hundreds of detected proteins could belong to dozens of pathways, be connected in different context-specific networks by protein interactions and be regulated by a variety of one-step and remote regulators (Bessarabova et al. 2012). Knowledge-based approaches deal with this complexity by creating a structured database of protein interactions and pathways, and a set of statistical tools to compare the proteomics profiles with this rich source of accumulated knowledge.

These knowledge bases include MetaCore (Thomson Reuters), IPA (Ingenuity), KEGG (Kanehisa and Goto 2000) and HPRD (Keshava Prasad et al. 2009), to name a few. The main types of data stored deal with protein functionality as represented by physical and functional protein interactions of different types (often assembled into multi-step pathways) and gene/protein-phenotype associations that link genes and protein variants to the diseases, toxic effects, drug responses, or other 'end points' of interest. Manually curated knowledge databases have rich semantics in a form of functional ontologies and controlled vocabularies of terms and synonyms. Genes, proteins, metabolic compounds and drugs are all assigned to particular entities, or terms, in multiple ontologies. For instance, cellular processes or standardised protein functions are all done by the GeneOntology consortium (http://www.geneontology.org/). Sub-categorisation of proteins and genes into ontologies and the representation of protein functionality as binary interactions (please see the 'network analysis tools' section for definitions of interactions) and multi-step pathways are the two pre-requisites essential for functional or 'knowledge-based' analysis. In general, the introduction of bioinformatics analysis to proteomics experimentation has ensured that repetition is avoided and that results are robust and reliable.

4.1.3 Application of Proteomics in LAB Studies

With LAB, proteomics approaches have been used in two main ways. First, they were used to reveal and identify the majority of proteins present in selected LAB on single 2-D gels called 'reference maps'. These studies aimed to provide information on the relative distribution and abundance of identified proteins from LAB species and isolates growing under controlled conditions (Champomier-Vergès et al. 2002). Preliminary results contributed to the establishment of 2-DE

images of proteins found in LAB and the identification and comparison of proteins in species/isolates from various origins including examples under certain metabolic processes such as stress. These first images can be regarded as a reference for subsequent research. Second, they were used to compare protein profiles from LAB submitted to different environmental challenges such as those that may be encountered during passage through the gut or during fermentation. The various stresses to LAB include acid, extreme temperature, salt stress, oxidative stress, high pressure stress and starvation. An understanding of differential expression of proteins during stress underpins the development of methods to enhance growth and/or survival in extreme environments. This information can also be applied to develop new food-grade starter cultures with stress tolerance properties using genetic engineering techniques (De Angelis and Gobbetti 2004).

4.2 Reference Map Construction

4.2.1 Proteomic Analysis of Whole-Cell Soluble Proteins

4.2.1.1 Lactococcus Lactis

Lactococcus lactis is the most studied species among LAB. While some species in the genera *Streptococcus* and *Enterococcus* are involved in human pathogenesis, species in the genus *Lactococcus* are usually isolated from dairy products and have a long history of safe use in food processing. Therefore, *Lactococcus lactis* has become a model organism in studies of growth physiology and gene function in LAB. The first 2-DE reference map of LAB emerged from research on *Lactococcus lactis*.

Although Hartke et al. (1996) analysed the whole-cell protein extracted from *Lactococcus lactis* isolate IL1403 (Hartke et al. 1996), they built the 2-DE map based on the traditional 2-DE procedures of O'Farrel (1975). The study focused on the acid tolerance response (ATR) of *Lactococcus lactis* and suggested that *Lactococcus lactis* adapted to lactic acid exposure in two different ways: a logarithmic-phase ATR, that may have be activated by protons and a stationary-phase ATR, that did not need activation by protons. This was an important result that informed subsequent stress studies.

Very soon after this, Kilstrup et al. (1997) described the first reference map for *Lactococcus lactis* subsp. *cremoris* isolate MG1363 using the Pharmacia 2-DE system with 11 cm dry strip Immobiline pH gradients (IPG) at pHs between 4 and 7 (Kilstrup et al. 1997). Of the 400–500 visible proteins, DnaK, GroEL and GroES heat shock proteins (HSPs); the HrcA (Orf1) heat shock repressor and the glycolytic enzymes pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase were identified using a combination of western blotting and direct N-terminal amino acid sequencing from the gels. At that time, 2-DE technology was still in a state of development and immature.

Subsequently an annotated reference map for *Lactococcus lactis* isolate NCDO 763 was constructed using easily reproducible conditions, and proteins were identified using both MS and N-ter sequencing (Anglade et al. 2000). This was the first study to suggest that a sonication step was necessary to prepare soluble proteins prior to 2-DE, because *Lactococcus lactis* is a Gram-positive bacterium. As before, pH gradients between 4 and 7 were able to resolve the majority of proteins and about 400 silver-stained spots were detected. Among randomly selected spots, 17 were identified, and 8 implicated in sugar metabolism. Moreover, the study chose a chemically defined medium (CDM) and halted culture growth at a specific, selected absorbance to improve the reproducibility of 2-DE patterns.

MRS and M17 are frequently used biochemical media for growth of species from the genera *Lactobacillus*, *Lactococcus* and *Streptococcus*. However, in order to understand the mechanisms involved in growth of LAB under different conditions, CDM have been used to provide experimental conditions that deliver more efficient protein radiolabelling for 2-DE techniques and the investigation of LAB stress responses (Savijoki et al. 2006).

After the 2.37 Mbp genome of *Lactococcus lactis* isolate IL1403 was sequenced (Bolotin et al. 2001), the structure of cytosolic proteins of *Lactococcus lactis* isolate IL1403 cultivated in M17 broth was resolved by 2-DE (using two pH gradients of pH 4–7 and 4.5–5.5) and analysed in more detail (Guillot et al. 2003). This study made it possible to describe, at the proteome level, a significant number of cellular pathways related to important physiological processes such as glycolysis, nucleotide metabolism, proteolysis, fatty acid and peptidoglycan synthesis, as well as technological processes such as fermentation. During this study, 239 different proteins were identified using MALDI-TOF/MS though PMF. Furthermore, 34 proteins were matched to open reading frames (ORF) for which there was no assigned function. The study also compared lactose-regulated proteins in two isolates of *Lactococcus lactis* at the proteome level.

4.2.1.2 Streptococcus Thermophilus

Within the LAB, *Streptococcus thermophilus* is one of the most widely used bacterium for the manufacture of yoghurt, cheese and other fermented food products. The properties of fermented products depend largely on the enzymic capacities of the species and isolates that make up the starter (Accolas et al. 1977). In order to separate and characterise the major proteins expressed by *S. thermophilus*, a 2-DE study was done on *S. thermophilus* isolate PB18 grown in M17 medium (Perrin et al. 2000). The silver-stained gels revealed approximately 270 spots distributed between pH 4 and 7. The soluble proteins from whole cells were fractionated in parallel using ammonium sulphate. Twelve proteins were purified by chromatographic techniques and 11 proteins in the 2-DE silver-stained gels were identified by Edman degradation. These proteins can now be used as internal markers. The study also compared proteomic profiles of two isolates of *S. thermophilus*, ST105 and PB18; 87 % of protein spots were common and specific

markers were existing. It is the first report of proteomic comparison of two strains in same species.

In a subsequent, more comprehensive study, the expression products of almost 200 different genes in *S. thermophilus* isolate PB18 were revealed using a proteomic strategy combining 2-DE plus MALDI-TOF/MS with differential 1-DE plus μLC-ESI-IT-MS/MS (Arena et al. 2006). A 2-DE reference map for lactose-grown cells was established using 18 cm of pH 4–7 linear IPG and pH 3–10 nonlinear IPG. 2-DE plus high-throughput MALDI-TOF/MS PMF separated cytosolic proteins and generated an annotated and quantified reference 2-DE map. The 1-DE plus μLC-ESI-IT-MS/MS analysis is essential to identify proteins that may be poorly soluble, and therefore not visible by 2-DE, or had a low-molecular mass. From 193 gene products, 53 were found using these two proteomic methodologies and 86 and 54 proteins were uniquely identified by 2-DE MALDI-TOF/MS and 1-DE μLC-ESI-IT-MS/MS analysis, respectively. Thus, a number of cellular pathways related to important physiological processes were described at the proteomic level. Proteomic variations associated with temperature, pH, oxidative and nutrient starvation stresses were also characterised. The study provided a better understanding of the biochemical processes related to stress resistance in *S. thermophilus*, allowing the authors to define the molecular basis of adaptative responses and reveal markers that facilitate the identification of particular isolates with potential industrial applications.

4.2.1.3 Species from the Genus *Lactobacilli*

Lactobacillus is the largest genus within the LAB. It contains a very large number of species isolated mainly from humans, animals, plants and foods (Stiles and Holzapfel 1997; Scheifer and Ludwig 1995). Since a few species have been identified as probiotics with human health benefits, their molecular metabolism has attracted a lot of attention. Technically, the short half life of mRNA can be a limiting factor in some studies. In contrast, proteins have the advantage of greater stability and proteome analysis of species in the genus *Lactobacillus* have elucidated important aspects of physiological processes and responses to stress (Cohen et al. 2006). Most research has focused on proteomic analysis of the growth phases of representative species, such as *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, *L. casei* and *L. acidophilus*.

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the most important industrial LAB because it is used, with *S. thermophilus*, in the production of yoghurt and fermented milk for human consumption. The proteome profiles of *L. delbrueckii* subsp. *bulgaricus* were first identified by Lim et al. (2000) as described previously for other species, using 2-DE gels and [^{35}S] methionine labelling (Lim et al. 2000). Three acid-induced proteins, GroES, GroEL and DnaK, were identified by N-terminal amino acid sequencing.

Lactobacillus plantarum is one of only a few *Lactobacillus* species that naturally inhabit the human gastrointestinal tract (GIT). It is predominantly found in the

small intestine varying from 10^6 to 10^8 colony forming units (CFUs) per gramme of mucosal biopsy (Ahrné et al. 1998; Reuter 2001). It has also been isolated from a variety of plant materials including cabbage, olives, *dajiang* (a kind of soybean paste made in northeast of China) and vegetables (Yoon et al. 2006; Lavermicocca et al. 2005; Wu et al. 2013). *L. plantarum* is an oxygen tolerant, heterofermentative species and can grow to high density which is desirable for industrial purposes. The complete genome of *L. plantarum* isolate WCFS1, from the human GIT, has been sequenced and 3,052 proteins in total have been predicted in either the cytosolic, membrane-bound or secreted form (Kuipers et al. 2003).

A more extensive proteome reference map of *L. plantarum* isolate WCFS1 from the cytosolic fraction of mid and late log, early and late stationary-phase cells grown on de Man, Rogosa and Sharpe (MRS) broth has been constructed; approximately 200 spots were identified using a pH range between 3 and 10 (Cohen et al. 2006). More than half (57 %) of the identified proteins were predicted to be involved in metabolic pathways. From these results, the most important reactions occurring during the log phase were concerned with generating sufficient energy. However, the highest rate of protein biosynthesis occurred during the late-log and early stationary phase and this is thought to be associated with the overall lower growth rate during this period. During the stationary phase, many stress proteins were induced in response to the unfavourable conditions of high acidity and diminished nutrients. Enzymes involved in the synthesis of cell wall structures were in greatest abundance during the stationary phase, presumably to strengthen the cell wall and to maintain the bacterial morphology. Furthermore, the downregulation of enzymes involved in glycolysis and the upregulation of galactokinase and UDP-glucose 4-epimerase during the early and late stationary phases suggests that the use of glucose as the main carbon source for energy generation via glycolysis was replaced by the Leloir pathway during these phases. The advantages of 2-DE technology were exemplified in this article.

Lactobacillus casei is a facultative heterofermentative LAB. Although it was first isolated from cheese, *L. casei* is widely distributed in various habitats, including fermented products, the intestinal tract, the oral cavity and even from soils and lakes (Randazzo et al. 2004). *L. casei* isolates are recognised as probiotics and have been used in numerous commercial products (e.g. *L. casei* Shirota isolate, *L. casei* Defensis isolate) for their beneficial and nutritional properties (Minellia et al. 2004; Coeuret et al. 2004).

In our research, we carried out a proteomic study to identify and characterise proteins expressed during the exponential and stationary growth phase by a probiotic isolate of *L. casei* (reference code Zhang) that had been isolated from traditionally made koumiss from Inner Mongolia (Wu et al. 2009a). Cytosolic proteins of this isolate cultivated in MRS were resolved by 2-DE using pH 4–7 linear gradients. The number of protein spots quantified from the gels was 487 ± 21 (exponential phase) and 494 ± 13 (stationary phase), respectively. Combined with genome information for this isolate (GenBank accession number CP00108 (Zhang et al. 2010a), a total of 131 spots were identified by MALDI-TOF/MS and/or MALDI-TOF/TOF according to significant differences in phase-

related growth or high expression intensity of some proteins. Followed by clustering of orthologous groups (COG), codon adaptation index (CAI) and GRAVY value analysis, the study provided the very first reference map for *L. casei* and an insight into the profile of protein expression.

Forty-seven spots were significantly different in their intensity between the exponential phase and stationary phase. Thirty-three of the spots were upregulated by at least 2.5-fold during the stationary phase compared with the exponential phase; this including 19 proteins (e.g. Hsp20, DnaK, GroEL, LuxS, pyruvate kinase and GalU) that were upregulated by 3.0-fold. Transcriptional profiles were made and real-time quantitative PCR (qPCR) used to confirm that several important proteins were differentially expressed. The analysis suggested that the differentially expressed proteins could mainly be categorised as stress response proteins and key components of central and intermediary metabolism. This indicated that these proteins may play a potentially important role in adaptation to surroundings, especially the accumulation of lactic acid during the course of growth.

Recently, the reference map of *L. acidophilus* isolate NCFM has been established using 2-DE at pH gradients between 3 and 7 (Majumder et al. 2011). This particular isolate is commonly used in dairy fermentations owing to its probiotic properties. In this study, with whole-cell proteome extracts from *L. acidophilus* NCFM grown on glucose until the late exponential phase revealed a total of 275 unique proteins (from 650 spots) that could be assigned to various physiological processes. Further, in a 2-DE (DIGE) comparison study at pH 4–7, it was found that the β-galactosidase small subunit, galactokinase, galactose-1-phosphate uridylyltransferase and UDP-glucose-4-epimerase were potentially involved in the prebiotic lactitol metabolism of this isolate.

The first report of a proteomic analysis of amine-producing bacteria, specifically *Lactobacillus* sp. isolate 30a and *Lactobacillus* sp. isolate W53 cultured in MRS medium, identified 22 spots in the pH range 4–7 during the exponential or early stationary growth phases (Pessione et al. 2005). These two isolates came from amine-contaminated wine and carried genetic determinants for histidine decarboxylase (HDC) and ornithine decarboxylase (ODC). Based on the results, HDC and ODC biosynthesis were shown to be closely dependent on the presence of high concentrations of free amino acids in the growth medium and to be modulated by the growth phase. The stationary phase and high amounts of free amino acids also strongly induced the biosynthesis of an oligopeptide transport protein belonging to the proteolytic system of LAB.

4.2.1.4 Species from the Genus *Bifidobacteria*

Species from the genus *Bifidobacterium* are anaerobic bifid or multiple branching Gram-positive rods that constitute one of the largest microbial populations in the GIT of humans. They can represent up to 91 % of the total gut microbiota, particularly in breast-fed infants, and *Bifidobacterium longum* is one of the most representative species (Harmsen et al. 2000).

The genome of *B. longum* isolate NCC2705 has recently been sequenced (Schell et al. 2002), prompting further investigations at the proteomic level including construction of a reference map. A comprehensive proteomic study identified and characterised proteins expressed by *B. longum* isolate NCC2705 using IPG at pH gradients 4–7, 4–5 and 4.4–5.5 (Yuan et al. 2006). A total of 708 spots representing 369 protein entries were identified by MALDI-TOF-MS and/or ESI-MS/MS. The identified proteins represented 21.4 % of the predicted 1727 ORFs in the genome and corresponded to 30 % of the predicted proteome. Moreover, 95 hypothetical proteins were experimentally identified. This was the first compilation of a proteomic reference map for this important probiotic species and also compared the proteomic profile of this isolate when grown on either fructose or glucose substrates; results indicated that intracellular fructose and glucose were catabolised via the same degradation pathway. However, sugar-binding proteins specific to fructose had a 10-fold higher expression level in cells grown on fructose than in cells grown on glucose. An ATP-binding protein of an ABC transporter was also slightly upregulated in cells grown on fructose compared with cells grown on glucose. It seems that the two proteins together play a role in fructose uptake in *B. longum* isolate NCC2705.

The 2-DE technique coupled with protein identification by MS has provided insights into the changes in protein abundance underlying basic biological processes. Although 2-DE is a powerful protein separation technique, it can be biased against proteins with extreme values of p*I* or *M*r (Corthals et al. 2000). To improve proteomics analysis, multidimensional proteins identification technology (MudPIT) method has been used for *B. infantis* (Vitali et al. 2005). The MudPIT technique consists of the sequential chromatographic separation of protein digests by SCX and reverse phase chromatography coupled with MS/MS (Link et al. 1999). One of the most significant features of the MudPIT approach is the capability to identify proteins with high mass and basic p*I* that are generally difficult to visualise using the 2-D SDS-PAGE format, for example, cell wall-associated proteins. The results of the study demonstrated this advantage; although the 136 proteins identified did not represent a complete analysis of the whole proteome of log phase cells of *B. infantis*, the method did provide a large-scale and global analysis of the proteome of this organism. The study also used the double chromatography technique to evaluate the much larger amounts of peptide data produced and identify redundancy, that could be considered as a disadvantage of the MudPIT method. However, it still represents one of the most promising alternative approaches to 2-DE for proteome characterisation.

4.2.2 Proteome Analysis of Alkaline Proteins of LAB

With respect to LABs with alkaline proteomes, there are very few available reference maps that include ranges for p*I*s > 7.0. In *Lactococcus lactis,* just over 56 % of the theoretical proteome is accounted for by acidic proteins with a

p*I* between 3.4 and 7, with only 43 % of the proteins displaying a p*I* > 7. In order to better understand *Lactococcus lactis* proteins with a predicted isoelectric point above 7, a reference map for alkaline proteins was established using IPG spanning pH 6–12 and 9–12, and proteins were identified by MALDI-TOF/MS (Drews et al. 2004). Alkaline proteomes are much more difficult to work with than acid proteomes because of the solubility properties of these proteins. The best 2-DE results were obtained by loading the sample on the anodic side; ultimately 153 proteins were identified from over 200 spots in the pH gradient from 6–12. Their predicted p*I*s ranged from as high as 11.31 to as low as 6.34. The largest groups of identified proteins comprised ribosomal proteins, hypothetical proteins and proteins with unknown function. Because the alkaline part of the proteome contains mainly hydrophobic proteins, no proteins associated with glycolysis were identified but most of the identified proteins were involved in translation or their function could not be predicted. Nonetheless, separation of the proteins between pH 6 and 12 provided an extension of the previously available reference map for *Lactococcus lactis* between pH 4 and 7.

More recently, a simpler and more standardised 2-DE protocol suitable for studying alkaline proteomes has been developed and used to study *L. hilgardii* from wine (Lamberti et al. 2007). Optimisation of the method focused on improving protein extraction and IEF (p*I* 6–11) separation protocols. The study tested and compared different methods for sample loading (e.g. in-gel rehydration and cup loading) and different reducing agents (e.g. DTT and bis (2-hydroxyethyl) disulfide (HED)). The results showed that a combination of cup loading, bis (2-hydroxyethyl) disulfide and protein precipitation protocols improved extraction of alkaline proteins from *L. hilgardii*. Moreover, despite differences in the external cell layers of species of *Lactobacillus* and *Acinetobacter*, the protocol resolved alkaline proteins efficiently from both groups.

An alkaline reference map for *L. acidophilus* isolate NCFM has also been established using 2-DE in pH gradients between 6 and 11 (Majumder et al. 2012). In this study, 150 proteins were identified from 202 spots picked from Coomassie Brilliant Blue-stained 2-DE gels and analysed using MALDI-TOF-MS. Reports on alkaline protein profiles fundamentally complement knowledge on protein profiles previously limited to the acid and neutral pH range.

4.2.3 Proteome Analysis of Cell Envelope Proteins of LAB

Considering the genome size of LAB is capable of encoding over 15,00–2,000 proteins, only about 50 % of these proteins can be detected through 2-DE, with those remaining, including membrane-associated proteins are difficult to identify using this technique because of solubility issues (Yuan et al. 2006; Gygi et al. 2000; Santoni et al. 2000). The importance of many cell envelope proteins for cellular homeostasis, pathogenesis and drug resistance makes them an obvious target for the development of novel therapeutics (Hurdle et al. 2011). Therefore,

proteomics techniques including LC-ESI-MS/MS have been developed and used to study membrane proteomes of LAB.

In the study of *Enterococcus faecalis*, a resident bacterium of the intestinal tract of humans and animals, membrane protein samples were separated using BN-/SDS-PAGE and 294 protein spots were detected in resulting reference maps (Maddalo et al. 2011). Analysis of these spots by nano LC-ESI-MS/MS indicated that they corresponded to 102 unique proteins associated with cellular homeostasis, virulence and antibiotic resistance. Intriguingly, many proteins with no known function were also identified, indicating that there are substantial gaps in knowledge about this organism's biology. This study represents the first step towards elucidating the membrane proteome of *E. faecalis*, a Gram-positive bacterium that is part of the indigenous microbiotica of humans and animals as well as an opportunistic pathogen.

Bifidobacterium animalis subsp. *lactis* isolate BB-12 is a widely used probiotic isolate associated with a variety of health-promoting traits. There is, however, only limited knowledge available regarding the membrane proteome and the proteins involved in oligosaccharide transport in isolate BB-12. Gilad et al. (2011a) applied two enrichment strategies to improve the identification of membrane proteins from isolate BB-12 cultures grown on glucose and on xylo-oligosaccharides (XOS), the latter being an emerging prebiotic substrate recently reported to be fermented by isolate BB-12 (Gilad et al. 2011a). The approach encompassed consecutive steps of detergent- and carbonate-treatment in order to generate 'inside-out' membrane vesicles and to interfere with binding of membrane-associated proteins to the membrane. Proteins in the enriched membrane fraction and membrane-associated fraction were digested by lysyl endopeptidase and trypsin followed by peptide sequencing. Ninety of a total of 248 proteins identified were unique and predicted to possess transmembrane segments (TMSs); 56 of these had more than one TMS. Seventy-nine of the identified proteins are thought to be involved in transport of amino acids, oligosaccharides, inorganic ions, nucleotides, phosphate or exopolysaccharides, or to belong to the F_1F_0-ATP-synthetase complex and the protein translocation machinery.

4.2.4 Secretome/Exoproteome of LAB

In bacteria, secreted proteins are transported via a variety of secretion systems to various locations, including being anchored to the cytoplasmic membrane, associating with the cell wall, released extracellularly, or injected into a host cell (Desvaux et al. 2009). Secreted proteins found in the extracellular milieu constitute the extracellular proteome, which is also known as the exoproteome or secretome (Curreem et al. 2012). These proteins contribute to bacterial adaptability to environmental change and play crucial roles in the infection process (Desvaux et al. 2010). For pathogenic species of bacteria, cell surface and extracellular proteins are increasingly being subjected to detailed proteomic investigation using

2-DE techniques, because the immunogenic properties of many proteins secreted by pathogenic bacteria make them prospective candidates for the development of subunit- or epitope-based vaccines (Gupta et al. 2009).

For the probiotic species *B. animalis* subsp. *lactis* isolate BB-12, secreted proteins were identified using 2-DE coupled with MALDI-TOF MS and their interactions with the host unravelled (Gilad et al. 2011b). Amongst the 74 distinct proteins identified, 31 were predicted to carry out physiological roles, either outside the cell or on its surface. These proteins included solute-binding proteins for oligosaccharides, amino acids and manganese; cell wall-metabolising proteins and 18 proteins that were described to interact with human host epithelial cells or extracellular matrix proteins. The potential functions include binding of plasminogen, formation of fimbriae, adhesion to collagen, attachment to mucin and intestinal cells, as well as induction of a host immune response. These findings suggest a role for the secreted proteins in colonisation of the GIT, adhesion to host tissues and modulation of the host immune system. The identification of proteins predicted to be involved in such interactions can pave the way towards well-targeted studies of the protein-mediated contacts between bacteria and the host, with the goal of enhancing our understanding of the mode of action of probiotic bacteria.

2-DE analyses were also used to compare the secretome of one food-associated *E. faecalis* isolate (DISAV 1022) and one clinical isolate of *E. faecalis* (H1) (Pessione et al. 2012). Patterns of extracellular proteins differed significantly between the two isolates, with only seven proteins being common to both. Notably, only the clinical isolate expressed various well-characterised virulence factors such as gelatinase coccolysin (GelE) and the extracellular serine proteinase V8 (SprE). Moreover, various other putative virulence factors, e.g. superoxide dismutase, choline- and chitin-binding proteins and potential moonlighting proteins, were detected exclusively in the secretome of the clinical isolate, but not in the food-associated isolate.

In conclusion, the examples described highlight how reference maps generated by proteomic techniques make important contributions towards understanding the metabolic activity of LAB during growth in standard media. Systematic analyses of the proteomes of LAB allow rapid identification of proteins and a basic understanding of the organism's metabolism on which to base future experiments. A different approach is to analyse proteins whose synthesis is induced by exposure to a variety of different environmental situations or stresses, which will be reviewed below.

4.3 Response of LAB to Environmental Stress

Over the years, many studies have aimed to better understand the response of LAB to environmental stress, particularly focusing on investigating the molecular metabolic factors involved in regulating LAB growth during passage through the GIT and during food processing. The environmental conditions considered mainly relate to extremes (both high and low) of pH, bile salts, temperature, salt, oxygen,

pressure and nutrient availability. It is essential to know not only which conditions are favourable or detrimental for the life of LAB but also the mechanisms that permit their survival and continued metabolic activity under stress conditions (De Angelis and Gobbetti 2004). The related proteomic studies of the stresses encountered by specific LAB species have generated increasing interest in recent years. The purpose of the following section is to review current efforts in proteomics research towards comprehending the physiological responses of LAB to acid, bile, heat, cold, osmotic, oxide and starvation stress.

4.3.1 Acid Stress

Lactic acid is formed as the end product of glycolysis in LAB when pyruvate is reduced to lactate and secreted into the culture medium (Ljungh and Wadstrom 2009). It means that, during growth, LAB face an environment that continually increases in acidity. The accumulation of lactic acid is a prerequisite for the fermentation of yoghurt, cheese, kimchi, sausage and other food products. It can also serve as a defence against other organisms that could spoil the food or be pathogenic.

Several theories for the mechanism of acid resistance have been reported for LAB (Foster 2000). These include homeostasis of intracellular pH by an F_1F_0-ATPase proton pump or the glutamate decarboxylase system; alkalization of the external environment with urease or arginine deiminase; repair of DNA and protein damage in general; physiological and metabolic pathway control via cell density and cell membrane changes and regulation (De Angelis and Gobbetti 2004; Foster 2000; Paul and Colin 2003). Recently, proteomic studies of LAB have been used to identify targets of the regulatory proteins involved in the acid stress response. The research can be divided into two types: studies of adaptive tolerance and studies of growth at low pH.

4.3.1.1 Adaptive Tolerance

There are at least two physiological mechanisms to increase acid tolerance (AT) in LAB: (a) during logarithmic growth an adaptive response referred to as L-ATR, which can be induced by incubation at a nonlethal acidic pH; (b) after entry into the stationary phase (Van de Guchte et al. 2002). Foster and Hall et al. (1991) first developed the concept of ATR which is an inducible mechanism to protect cells from acid death (Foster and Hall 1991).

Logarithmic Phase Adaptation (L-ATR)

Most of the LAB species tested possessed an L-ATR, except for many isolates of *Lactococcus lactis* subsp. *cremoris* (Kim et al. 1999). Numerous proteomic studies on L-ATR showed that a large number of proteins were induced during acid

adaptation in LAB (Champomier-Vergès et al. 2002). These findings provided new insights into the inducible mechanisms facilitating LAB tolerance to acid stress.

In *Lactococcus lactis*, acid adaptation in *Lactococcus lactis* subsp. *cremoris* isolates MG1363 and 712 both require de novo protein synthesis. In contrast, acid adaptation in *Lactococcus lactis* subsp. *lactis* isolate IL403 is reported to be independent of protein synthesis, although induction of UV-inducible proteins and HSPs are observed (Hartke et al. 1996, 1997). HSPs and ClpATP were also induced by acid in *L. rhamnosus* isolate E800, *L. brevis* isolate ATCC 8287 and *L. reuteri* isolate E849 (Savijoki et al. 2006), as well as the Zhang isolate of *L. casei* (Wu et al. 2011). GrpE was over-expressed in acid-adapted and acid-tolerant *L. sanfranciscensis* mutants compared with nonadapted cells (De Angelis et al. 2001). GroES, GroEL and DnaK were identified in acid-adapted *L. bulgaricus* cells (30 min incubation at pH 4.75) which were 250-fold more tolerant to lethal acid stress (30 min at pH 3.6) than nonadapted cells (Lim et al. 2000). Besides the obvious stress proteins, other proteins involved in metabolic pathways were also differentially regulated in response to acid stress.

Differential L-ATR has also been observed in *Lactococcus lactis* isolate MG1363 when incubated at pH 5 in either a rich medium (M17) or a CDM (SA) (Budin-Verneuil et al. 2005). 2-DE revealed a total of 90 unique spots induced by acidity, 80 of which were identified by MS. Only 10 proteins (BglA, PycA, GlmS, HasC, ArgS, GatA, AtpA, ArcB, Cfa and SodA) were over-expressed in the two media. Amongst the proteins over-expressed during the L-ATR in SA but not in M17, 13 already displayed an elevated rate of synthesis in M17 at a neutral pH. These proteins could play an important role in the development of the protein de novo synthesis-independent L-ATR observed in M17.

In a protein synthesis study of the probiotic species *L. reuteri*, cells at the mid-exponential growth phase were exposed to transient low-pH stresses from pH 6.8 to 5.0, 4.5, or 4.0 in 1 h or pH 3.0 in 1.5 h at 37 °C (Lee et al. 2008; Lee and Pi 2010). Forty and 16 common proteins were identified by 2-DE and PMF, respectively. Functional annotation showed they were associated with metabolism, transport and binding proteins, transcription–translation, pH homeostasis metabolism, DNA replication/repair and stress responses.

2D-DIGE was used to study the global proteome of the probiotic species *L. rhamnosus* isolate GG to two physiologically relevant pH conditions (pH 4.8 and 5.8). In total, 2D-DIGE revealed 92 pH-dependent proteins, and the greatest upregulation of all proteins was detected, as predicted, for the surface antigen, LGG_02016 (Koponen et al. 2012). In addition, the proteomics data were complemented with transcriptome analyses using whole-genome DNA microarrays, and showed that upregulation of F_0F_1-ATP synthase genes and over-expression of their associated proteins occurred at the acid-pH, whereas the abundance of proteins participating in nucleotide biosynthesis and protein synthesis was significantly diminished. Moreover, the results suggested that *L. rhamnosus* isolate GG modulates its pyruvate metabolism depending on the pH.

Further, proteome comparison of LAB isolate and its acid-resistant mutant is one of effective ways to reveal the acid adaptation mechanisms.

Analysis of physiological data from a comparative physiological and proteomic study of lactic acid-induced alterations in the *L. casei* Zhang isolate and its acid-resistant mutant showed that the mutant exhibited 33.8 % higher activity in glucose phosphoenolpyruvate and the sugar Phosphotransferase system and a lower glycolytic pH compared with the wild-type under acidic conditions (Wu et al. 2012). Comparison of the proteomic data based on 2D-DIGE indicated that acid stress induced a global change in proteins in both isolates. Interestingly, the wild-type had higher levels of intracellular aspartate and arginine, and achieved a 1.36- and 2.10-fold improvement in survival at pH 3.3 by the addition of 50 mM aspartate or 50 mM arginine, respectively. So it was the first demonstration that aspartate may be involved in AT in *L. casei*.

The mechanisms underlying responses to acids and adaptation in *B. longum* isolate NCIMB 8809 and its acid-pH-resistant mutant isolate 8809dpH were studied (Sánchez et al. 2007a). Comparison of protein maps constructed by 2-DE and protein identification by MALDI-TOF/MS analysis, allowed nine proteins to be identified that were differentially expressed in the mutant isolate. Furthermore, the production of 47 proteins was modulated by pH in one or both isolates. These included general stress-response chaperones and proteins involved in transcription and translation as well as in carbohydrate and nitrogen metabolism, amongst others. Significant differences in the levels of metabolic end products and in the redox status of the cells were also detected between the wild-type isolate and its acid-pH tolerant mutant in response to, or as a result of, adaptation to acid. Remarkably, the results of this work indicated that adaptation and responses to low pH in *B. longum* biotype *longum* involve changes in the glycolytic flux and in the ability to regulate the internal pH. These changes were accompanied by a higher content of ammonium in the cytoplasm, likely to be derived from amino acid deamination and a decrease in the hydrolase activity of bile salt.

In addition to lactic acid stress, researchers have also considered acid stress caused by other organic acids, such as tannic acid (TA) which occurs naturally in wine. The interaction between TA and *L. hilgardii*, a LAB associated with wine spoilage was investigated using a combination of physiological and proteomic approaches (Bossi et al. 2007). Total proteins extracted from unexposed and TA-exposed cells were analysed by 2-DE and significant reductions in protein spot intensity when exposed to TA were observed. Most of the proteins, identified by ESI-MS, were metabolic enzymes from different pathways in the cytoplasm and cell membranes. Recently, a proteomic analysis of a human-derived *L. plantarum* isolate exposed to TA showed that cells of *L. plantarum* challenged with TA reorganised their metabolic capacity to save energy and expressed proteins involved in oxidative stress defences and cell wall biogenesis (Curiel et al. 2011). This indicated that injury incurred by TA was based on oxidative damage and disruption of the cell wall envelope. Induction of 3-octaprenyl-4-hydroxybenzoate carboxy-lyase, which is sensitive to changes in redox conditions and involved in ubiquinone biosynthesis in other bacteria, suggests a TA-induced redox imbalance in *L. plantarum*.

Stationary-Phase Adaptation and Starvation

After entering the stationary phase, AT ability commonly increases as a result of the induction of a general stress response. In *Lactococcus lactis* isolate CNRZ 157, proteomics and transcriptomics studies showed that DnaK was induced in the stationary phase in response to increased lactic acid concentrations (Larsen et al. 2006). In *L. acidophilus*, the survival of stationary-phase cells under acid stress is a function of the low pH attained by the cultures. The study of protein profiles showed that seven proteins were overexpressed as a result of the stationary phase itself, while nine proteins were exclusively induced as a result of the drop in pH during fermentation. Thus, stationary-phase ATR results from a combined effect of the pH and concentration of lactic acid in the medium (Lorca and de Valdez 2001). Moreover, in *S. thermophilus* isolate PB18, a 16 kDa protein was over-expressed in the stationary phase as well as 2 h after an acid shock; furthermore, the protein was not expressed when the bacteria reached the stationary phase in media with low concentrations of lactose (5 or 10 $g l^{-1}$), in which the pH (5.5) was not as acidic as in the control cultures (pH 4.7, lactose 20 $g l^{-1}$). The results support the idea that this protein is expressed in response to the acidic environment and not in response to the growth phase (González-Márquez et al. 1997).

In fact, it should be noted that, when cells enter the stationary phase, several factors affect their development simultaneously: starvation, pH, oxygen availability (Champomier-Vergès et al. 2002). They are thus submitted to multiple stresses at the same time making it particularly difficult to determine the nature of the inducing stimulus for each protein.

2-DE techniques have been used to study the response of *E. faecalis* during the stationary growth phase to physical and chemical stresses as well as to glucose and to total starvation (Giard et al. 2001). Twenty-seven protein spots on 2-DE gels were identified by N-terminal sequencing and Western blotting to provide the first proteome database for this species. The proteins were classified into four groups according to their function. The first group was comprised of well-characterised proteins with known protective functions in response to stress. The second group contained enzymes from catabolic pathways but their role in stress tolerance was not obvious. A third group was comprised of proteins induced in glucose-starved cells and included the CcpA protein involved in carbon catabolite repression. Induction of these enzymes under starvation conditions may serve to increase the scavenging capacity of the cells for nutrients or may be important in mobilising endogenous energy reserves. The final group contained nine N-terminal amino acid sequences or ORF with no homologies with any sequences from existing databases.

Interestingly, once the stationary phase was reached, *Oenococcus oeni* isolate VP01 from wine showed a 99 % reduction in cell viability within 4 days. The remaining cell population maintained viability for 70 days and, when plated on agar medium, generated small colonies (Zapparoli 2004). The occurrence of this phenomenon was associated with stress tolerance, since 10 days old cells were also more resistant than 3 days old cells to ethanol and low-pH conditions. Total

protein analysis by bidimensional electrophoresis highlighted different protein expression in cultures of different ages. It was also shown that starving *O. oeni* cultures at the stationary-phase-retained dynamic cell populations. These results provide an interesting perspective on cell behaviour when inoculated into wine.

Cross-Tolerance

Both L-ATR and stationary-phase adaptation during starvation not only protect LAB from acid challenge but also from other stresses such as heat, cold and osmotic or oxidative shocks. Research of *L. delbrueckii* subsp. *bulgaricus* isolate CFL1 showed that cells acidified at the end of fermentation (pH 5.25 for 30 min) had their cryotolerance improved compared to cells under standard conditions (pH 6.0) (Streit et al. 2008). This was referred to as a cross-protection phenomenon as it meant acid-adapted cells were also more tolerant to cold stress. Analysis of the cytosolic proteome showed that changes occurred in the synthesis of 21 proteins involved in energy metabolism, nucleotide and protein synthesis and stress responses. Acidification also induced a slight decrease in unsaturated to saturated and cyclic to saturated fatty acid ratios in membranes. Hence this species was able to develop a combined physiological response at both membrane and cytosolic levels. This may be useful for improving cryotolerance in LAB, either in cells banks or in an industrial context.

It is noteworthy that the broad protective effects of L-ATR varies between species and does not always protect from the same sets of stresses (Quivey et al. 1995; Flahaut et al. 1996b; Svensater et al. 2000). For example, tolerance to stress and cross-protection in *L. collinoides* were examined after exposure to ethanol, acid or heat shock (Laplace et al. 1999). Ethanol and heat-adapted cells demonstrated induced homologous cross-tolerance to acid stress. However, no cross-protection against ethanol and heat stress was observed in acid-adapted cells. Heat was the only pretreatment leading to cross-protection against the other two stresses. Analysis of whole-cell protein extracts revealed that each treatment induced a battery of stress proteins and the synthesis of some of these polypeptides was induced by more than one stress factor. The greatest overlap was observed between ethanol and heat treatments. Ten proteins were found to be common to these two stresses.

4.3.1.2 Growth Under Acid Conditions

For LAB, it is special that the pH value of the growth environment keeps decreasing by the end products of LAB. The initial pH is important for the growth of LAB and may be in charge of other properties, e.g., anti-allergy. In recent research, the study showed that the decrease in pH from 5.0 to 4.5 during the growth of probiotic strain *L. acidophilus* isolate L-92 had greatest effect on the release of IL-12, IFN-γ and IL-10 (Kuwana and Yamamoto 2012). Comparative

proteomics analysis between L-92 cells cultured at pH 5.0 and 4.5 in MRS medium revealed that the expression of cell wall-associated components GroES and GroEL increased at lower pH. Transcriptome analysis also found 121 genes were significant upregulation and 92 genes were downregulated, including genes of GroES and GroEL. Hence, it was considered that the HSP might be the most likely components affecting immunomodulation.

Beside, a few reports have investigated the mechanisms underlying the ability of oral pathogen bacteria, *Streptococcus mutans* or *Streptococcus oralis*, to survive and proliferate at low pH by culturing them at pH 5.2 or 7.0 using 2-DE and MALDI-TOF/MS (Wilkins et al. 2001, 2002). Len et al. (2004) also used differential 2-DE proteome to analyse *S. mutans* grown at a steady state in continuous culture at pH 7.0 or 5.0 (Len et al. 2004). The induction of HSPs was found which might be responsible for the growth under acid condition, including 60 kDa chaperone, Hsp33, Hsp70, etc.

Overall it was apparent that a complex network of proteins was induced by acid stress in LAB and further research is required to fully understand this.

4.3.2 Bile Stress

After passing through the stomach, the next stress encountered by LAB is bile in the small intestine, which they must survive in order to transit or colonise the intestine. The relevant physiological concentrations of human bile range from 0.3 to 0.5 % (w/v) (Dunne et al. 2001; Zavaglia et al. 1998). In general, Gram-positive bacteria seem to be more sensitive to the deleterious effects of bile than Gram-negative bacteria (Begley et al. 2005). In one study, the minimum inhibitory concentrations (MICs) of ox gall (=bovine bile) to kill *Salmonella typhimurium* and *Salmonella typhi* at the stationary phase were 18 and 12 % (w/v), respectively (Van Velkinburgh and Gunn 1999). In another study, by Jacobsen et al. (1999) most *Lactobacillus* species examined (31 out of 47) did not replicate in broth supplemented with 0.3 % ox gall (w/v), although there was isolate to isolate variation in tolerance to bile salts (Jacobsen et al. 1999). MICs of ox gall for seven species from the genus *Bifidobacteria* ranged between 1.0 and 2.0 %, while for a further 12 species tested they were below 1.0 % (Margolles et al. 2003).

To emulsify fats and promote fats digestion is the main function of bile in vivo. Therefore, it may give an important role of bile to defence against micro-organism in body's intestinal tract (Zavaglia et al. 1998). Bile also functions as an excretory fluid by eliminating substances that cannot be efficiently excreted in urine because they are insoluble or protein bound, e.g. cholesterol, which is derived from excess synthesis or from the pigment bilirubin (Hofmann 1999). Ox gall is commonly chosen to assess the in vitro bile tolerance of bacteria. Proteomics analysis is a powerful tool for global evaluation of protein expression and has been applied widely to analysis of the molecular mechanisms involved in bile salt tolerance in LAB. Combined 2-DE and MALDI-TOF MS have contributed greatly to current

understanding of the mechanisms of bile salt tolerance in LAB and revealed a number of targets and molecular markers for future genetic and physiological studies as described below.

4.3.2.1 General Stress Responses

In previous research, stimulation of the expression of HSPs in response to the presence of bile salts has been reported in several LAB (Flahaut et al. 1996a; Savijoki et al. 2005; Sánchez et al. 2005; Wu et al. 2010). HSPs come from a number of different molecular groups including the DnaK (70 kDa) and GroEL (60 kDa) families, and are known to function as chaperones, participating in cellular functions such as protein folding, protein stabilisation and protein turnover. Activity of the sHsp group of HSPs is confined to the binding of unfolded protein intermediates to protecting them from irreversible aggregation. The function of sHsp is not dependent on ATP, unlike other HSPs such as Hsp70. In *E. faecalis*, *B. longum* and *L. casei* (Zhang isolate), DnaK and GroEL were over-expressed in response to bile salts (Flahaut et al. 1996a; Savijoki et al. 2005; Sánchez et al. 2005; Wu et al. 2010). In *Propionibacterium freudenreichii*, DnaK and Hsp20 were both over-expressed in response to bile salts (Leverrier et al. 2003).

Clp proteases are involved in maintaining the quality of cellular proteins (Frees et al. 2007) and assist molecular chaperones in counteracting the deleterious effects of bile (Burns et al. 2010; Whitehead et al. 2008). However, the Clp proteases of *L. johnsonii* isolate PF01, specifically ClpE, ClpA, HslV (ClpQ) and HslU (ClpY), were downregulated in response to bile (Lee et al. 2013), compared to the Clp proteases in other LAB species, such as *L. delbrueckii* subsp. *lactis*, *L. acidophilus* and *L. reuteri* which were not found. Furthermore, the peptidase subunit of ClpYQ (spot 26, LCAZH_1390) was over-expressed in *L. casei* (Zhang isolate) by 1.5 % ox gall (Wu et al. 2010). However, although the activity of these proteases is affected by bile salts, their role in LAB remains unknown.

4.3.2.2 Cell Envelope-Related Functions

As mentioned above, bile salts have great effect on the damage of bacterial membranes. The cell morphological changes in *P. freudenreichii* isolate SI41 by bile salts have been observed and reported (Leverrier et al. 2003). Cells grown under standard conditions exhibited the characteristic pleomorphic rod-shaped morphology of dairy propionibacteria. Their average length was 1.63 ± 0.28 μm (mean and standard deviation). However, incubation for 1 h in 1.0 g of bile salts/litre (challenge conditions) caused drastic changes in cell morphology. On the contrary, the alteration of membrane characteristic of bacteria by acid adaptation in the increasing of osmolarity possibly stumilates the resistance to bile salts (Begley et al. 2002). Proteomics data of the response of *B. animalis* subsp. *lactis*

isolate IPLA 4549 and its bile-resistant mutant *B. animalis* subsp. *lactis* isolate 4549dOx to bile salts also supported that the hypothesis that cell envelope functions are influenced by bile salts due to the proteins changes in fatty acid biosynthesis (Sánchez et al. 2007b).

Lactobacillus rhamnosus isolate GG is a widely used probiotic bacterium and also is able to be isolated in human intestinal tract (Boyle et al. 2009). Although the health benefits of isolate GG are well documented, a systematic exploration of the mechanisms by which this isolate exerts its probiotic effects in the host have only recently been initiated. An ability to survive in the harsh conditions (gastric juice and bile salts) of the GIT is essential for any probiotic bacterium to colonise within the host. Gene expression profiling at the transcriptome and proteome levels were used to investigate the cellular response of strain GG to bile under defined bioreactor conditions (Koskenniemi et al. 2011). The analyses revealed that during growth of isolate GG in the presence of 0.2 % ox gall the transcript levels of 316 genes changed significantly and 42 proteins, including both intracellular and surface-exposed proteins (i.e. surfome), were differentially expressed. The changes in protein abundance were correlated with transcriptome level changes for 14 of these proteins. The proteins identified suggest diverse and specific changes in general stress responses as well as in cell envelope-related functions, such as in the pathways affecting fatty acid composition, cell surface charge and thickness of the exopolysaccharide layer. These changes are likely to strengthen the cell envelope against bile-induced stress. Notably, the surfome analyses demonstrated significant reductions in the abundance of a protein catalysing the synthesis of exopolysaccharides, whereas a protein dedicated to active removal of bile compounds from the cells was over-expressed. These findings suggest a role for these proteins in facilitating the well-known beneficial interactions between isolate GG and host mucus in the presence of sublethal doses of bile.

To address how bile can influence the cell envelope proteome of *B. longum* isolate NCIMB 8809, Ruiz et al. (2009a) analysed its membrane protein using stable isotope labelling of amino acids in cell culture (SILAC) (Ruiz et al. 2009a). As a result, 141 proteins were identified in the membrane fraction, including a large proportion of theoretical transporters. Moreover, the envelope-associated soluble fraction was analysed using different sub-fractionation techniques and DIGE. This approach identified 128 different proteins. Some were well-known cell wall proteins but others were highly conserved cytoplasmic proteins probably displaying a 'moonlighting' function. The study identified 11 proteins in the membrane fraction and six proteins in the envelope-associated soluble fraction whose concentration varied in the presence of bile. Further, bile also promoted changes in the abundance of proteins with other important biological functions, such as some ribosomal proteins and enolase. Furthermore, oligopeptide-binding proteins accumulated on the cell surface, which was reflected in a different tripeptide transport rate in the cells grown in the presence of bile. The data may contribute to understanding bile tolerance in these bacteria.

4.3.2.3 Carbohydrate Metabolism Proteins

The proteomic profile for *B. longum* isolate NCIMB 8809 under intestinal stress conditions detected 34 protein spots on 2-DE gels using the genome sequence of *B. longum* isolate NCC2705 (Sánchez et al. 2005). The intensity of these spots increased or decreased depending on the presence of bile salts, mimicking the bile stress encountered in the human GIT. These data clearly demonstrate that the expression of proteins from different functional categories is modulated as a result of the exposure of *B. longum* to bile salts. Some of these proteins were identified as general stress-response proteins, but others were key components of the central and intermediary metabolism, including the transcription–translation machinery, gene regulation and protein synthesis. Remarkably, xylulose 5-phosphate/fructose 6-phosphate phosphoketolase, the key enzyme in the so-called 'bifidobacterial shunt', was over-expressed, and the activity on fructose 6-phosphate was significantly greater in protein extracts from cells grown in the presence of bile. Changes in the levels of metabolic end products (acetate and lactate) were also detected. The activation of glycolysis and pyruvate catabolism suggests a shift in bacterial metabolism to render more reducing equivalents and energy-rich intermediates, such as ATP. Besides, adenylate kinase, an enzyme involved in adenine synthesis and conversion of AMP to ADP was also found in larger quantities when bile salts were present in the medium. Similar results for changes in expression of Xfp in relation to bile salts was also recorded in *B. animalis* (Sánchez et al. 2007b).

4.3.2.4 Amino Acid Biosynthesis Proteins

Research in our laboratory showed that *L. casei* (Zhang isolate) had the ability to grow in 1.5 % ox gall and has greater tolerance to bile salts than other *Lactobacillus* species isolated from koumiss (Wu et al. 2009b). The results of electrophoresis indicated that multiple metabolic pathways might be involved in the adaptation of this isolate to the stress of bile salts, as the expression of 26 proteins with various functions were influenced by the presence of bile salts. We hypothesise, from results of qRT-PCR experiments, that proteins involved in cell protection, modifications in cell membranes and key components of central metabolism may play an important role in tolerance to bile salts in *L. casei* (Zhang isolate) (Wu et al. 2010). Previous studies have shown that over-expression of CysK (spot 14, 21, LCAZH_0511), played a role in protection against oxidative stress in response to bile salt stress (Leverrier et al. 2003); its levels increased by over 3.0-fold during growth of *L. casei* (Zhang isolate) in 1.5 % bile salts. Bile salts may cause oxidative stress by the generation of oxygen free radicals (Payne et al. 1998).

2-DE analysis of *P. freudenreichii* revealed 24 distinct spots from gels that were associated with protein protection/degradation, heat shock, oxidative stress, signal sensing and transduction and an alternative sigma factor. When *P. freudenreichii* was subjected to acid, heat and bile salt stress, six proteins were found to be over-

expressed in common, of which SodA was considered to possibly have a disoxidation effect (Leverrier et al. 2004).

Cysteine-derived proteins, such as thioredoxin and glutathione, have the ability to maintain the reducing state in bacteria (Sperandio et al. 2005; Lebeer et al. 2008). As in *Lactococcus lactis* and *L. plantarum* (Van de Guchte et al. 2002; Sperandio et al. 2005; Bron 2003), we have found that cysK is located with metC in *L. casei* (Zhang isolate) (unpublished data). The mRNA expression of metC in response to bile salt treatment in *L. casei* is also upregulated as determined by qRT-PCR (unpublished data). Thus, the metC-cysK operon, which is involved in cysteine and methionine metabolism, may be related to tolerance of *L. casei* to bile salt stress. Moreover, the upregulation of LuxS protein (spot 24, LCAZH_0709), which has been reported previously to be involved in the synthesis of cysteine from methionine in *L. casei* (Irmler et al. 2008), was also observed in this study. It is clear that this research is at an early stage and further work is needed to study the relationship between the enzyme CysK and bile salt tolerance.

Our results also showed that two peptidases, Pep Q (spot 23, LCAZH_1633) and PepC (spot 25, LCAZH_2303), were reduced in expression in response to bile stress. PepC is a cysteine aminopeptidase and was repressed in *L. casei* (Zhang isolate) when exposed to 1.0 % (w/v) ox gall for 30 min (Wu et al. 2010). Although Zaidi et al. (2008) reported that of the extensive proteolytic system of pepC in *Lactococcus lactis* exhibited significant repression during cholate stress, the changes in peptidases in *Lactobacillus* species have seldom been reported in previous studies (Zaidi et al. 2008).

4.3.2.5 Bile Salt Hydrolases

Bile salt hydrolases (BSHs) are enzymes (EC 3.5.1.24) that catalyse the hydrolysis of the amide bond between the C-24 position of the steroid moiety and the amino acid side chain of bile acids (Begley et al. 2005; De Boever et al. 2000). BSHs are generally intracellular enzymes that are oxygen insensitive, have a slightly acidic optimal pH (usually between pH 5 and 6), their activity is coupled to biomass production and they are not regulated by bile salts.

The BSH of *L. johnsonii* isolate 100–100 was inducible by bile (Lundeen and Savage 1992). Indeed, the activity of BSH in this isolate of *L. johnsonii* increased 3–5-fold within 20 min after conjugated bile salts were added to stationary phase cells. Lee et al. (2013) examined the growth inhibition, surface morphology and physiological aberrations in a naturally bile salt tolerant isolate (PF01) of *L. johnsonii* (Lee et al. 2013). This was the first time bile tolerance in this isolate of *L. johnsonii* was evaluated by investigating the morphology and growth rate of cells exposed to bile salts at concentrations of 0.1, 0.3 and 0.5 %. Cells grown in bile-free MRS medium were rod-shaped and had smooth surfaces, as compared to cells grown overnight in medium containing 0.3 % bile, which displayed a rough, shrunken and empty appearance. Further, quantitative proteomic profiles using iTRAQ–LC–MS/MS technology on the same isolate identified 8,307 peptides

from both untreated cells and those exposed to 0.1, 0.2 and 0.3 % bile salts. Of these, 215 proteins changed in response to bile stress; of these, levels of 94 induced while those of 121 reduced in expression. In particular, three BSHs (BSHA, BSHB and BSHC) identified by proteomic analysis were significantly upregulated in response to bile stress. BSHB has previously been shown to hydrolyse tauroconjugated bile salts while BSHC has hydrolytic activity against glycoconjugated bile salts and BSHA has similar properties as BSHB in terms of bile salt specificity (Oh et al. 2008). Thus, *L. johnsonii* isolate PF01 can hydrolyse all types of bile salts; this provides a distinct advantage in terms of survival in the GIT, compared with species and isolates that can only hydrolyse either glycoconjugated or tauroconjugated bile salts.

In addition, ribosomal proteins, transcription and translation proteins and nuclear metabolism proteins have been observed in proteomic studies of LAB, showing their important role in tolerance to bile salts. Thus, rather than a single mechanism, bile salts induce a complex physiological response to which proteins of a variety of functional categories contribute.

4.3.3 Adhesion

The main criteria for selecting probiotic isolates are their tolerance to acid and bile which facilitates their survival through the GIT, their ability to adhere to and colonise intestinal surfaces, their antagonism against pathogens and their technical ease of production (Salminen et al. 1996). The in vitro adhesion properties of probiotic LAB have been studied using different techniques including ELISA (Roos et al. 2000), radioactive labelling with ^{3}H (Ouwehand et al. 2001), diagnostic microscopy following methylene blue staining (Edelman et al. 2003), fluorescein isothiocyanate labelling (Edelman et al. 2003) and PCR (Nitisinprasert et al. 2006). Proteome analysis could provide further insights to the mechanisms involved.

Lactobacillus salivarius isolate UCC118 has been isolated from human intestinal digestive tract and has been regarded as a probiotic (Neville and O'Toole 2010). The isolate UCC118 has the ability to adhere to animal and human intestinal tissues. Proteomic analysis and enzymatic techniques have been combined to correlate bacterial growth phase with the presence of factors present in the cell wall associated with adhersion (Kelly et al. 2005). Using 1-D PAGE electrophoresis, a 84 kD protein that was associated with in vitro adhesion ability was found based on the comparison of the proteins isolated from lag to log to stationary growth phases. Further, the 84 kD band was separated by 2-DE and MS to identify the proteins involved. The study found 20 individual protein spots at differing isoelectric points, included DnaK, Ef-Ts and pyruvate kinase.

Using 2-DE and MS analysis key proteins in the adhesion of *L. fermentum* isolate I5007 and *L. plantarum* have been reported. For the *L. fermentum* isolate, in vivo and in vitro models were used: the isolate was either inoculated into rabbit

jejunum for 4 h or cultured in vitro with Caco-2 cells for 1 h (Yang et al. 2007). The results indicated that, after exposure to the intestinal environment, key enzymes involved in energy metabolism (e.g. lactate dehydrogenase, dihydrolipoamide dehydrogenase and nicotinate phosphoribosyltransferase) and amino acid metabolism (e.g. arginyl-tRNA synthetase and aspartate-semialdehyde dehydrogenase) decreased in quantity, but levels of glycoside hydrolase (an enzyme for mucin degradation) and fructose-6-phosphate phosphoketolase (an enzyme of the pentose phosphate pathway) increased in quantity. In response to an interaction with the *L. fermentum* isolate the in vitro Caco-2 cells showed changes in the proteins present that were beneficial for gut integrity, including voltage-dependent anion channel 1, glutathione transferase, and HSP gp96. In *L. plantarum*, cell wall extracts were subjected to proteomic analysis of differential protein expression as visualised by 2-DE (Izquierdo et al. 2009). Elongation factor EF-Tu, GroEL chaperone, molecular chaperone DnaK and GAPDH were over-expressed in the cell wall proteome of the highly adhesive isolate of *L. plantarum* reference WHE 92.

Glyceraldehyde-3-phosphate dehydrogenase has also been detected by Martín et al. (2012). In the study, the adhesion to mucin of 43 isolates of human-derived *Lactobacillus* species was analysed and the most adherent isolates were selected. Further, the components of the extracellular proteome of all isolates were identified by MALDI-TOF/MS. Except for GAPDH, a collagen-binding A precursor and aggregation-promoting factor-like proteins were identified and it was suggested that they participated in adhesion to Caco-2 and HeLa cells, respectively.

4.3.4 Heat Stress

The industrial preservation of LAB involves processes such as air-drying. These processes can lead to heat shock that results in structural and physiological injury to the bacterial cells and substantial losses of viability (Prasad et al. 2003). The effect of heat shock and the induction of a stress response in *Lactobacillus* spp. have been studied for *L. delbrueckii* subsp. *bulgaricus*, *L. paracasei* and *L. salivarius* (Desmond et al. 2001; Gardiner et al. 2000; Gouesbert et al. 2001), *L. acidophilus*, *L. casei* and *L. helveticus* (Broadbent et al. 1997), *L. collinoides* (Laplace et al. 1999), *L. sakei* (Schmidt et al. 1999), *L. johnsonii* (Zink et al. 2000) and *L. plantarum* (Lee and Kaletunc 2002; Smelt et al. 2002; Jordan and Cogan 1999).

The major problem encountered by cells at high temperature is the denaturation of proteins and their subsequent aggregation (Somero 1995). In addition, destabilisation of macromolecules such as ribosomes and RNA, and alterations in membrane fluidity have also been described (Earnshaw et al. 1995; Teixera et al. 1997; Hansen et al. 2001).

The major impact of the HS response as revealed by proteomic analysis of LAB is the induction of a set of chaperones and proteases that both contribute to the process of refolding or elimination of damaged proteins (Champomier-Vergès et al. 2002). However, studies of *L. plantarum, L. rhamnosus* and *L. helveticus* also

showed that heat resistance was a complex process involving proteins with a variety of roles in cell physiology, including chaperone activity, glycolysis-related machinery, ribosome stability, stringent response mediation, temperature sensing, control of ribosomal function and other regulatory processes (Prasad et al. 2003; Di Cagno et al. 2006; De Angelis et al. 2004).

In order to analyse the thermal adaptation mechanisms involved, thermoresistant variants of *L. delbrueckii* subsp. *bulgaricus* were selected (Gouesbet et al. 2002). These variants showed enhanced constitutive tolerance towards heat shock. However, contrary to the wild-type isolates from which they were derived, these variants were poorly protected after either osmotic or heat pretreatments. This result suggests that, above a certain threshold, cells reach a maximum level of protection that cannot be easily exceeded. A comparison of protein patterns showed that the variants were able to induce the proteins involved in their adaptive mechanisms more rapidly than the wild type. In particular, the variants were able to express constitutively more HSP, leading to the higher level of thermal protection.

Using 2-DE and efficient protein radiolabelling tools, protein synthesis in *B. longum* cells before and after heat shock and bile salt treatment was investigated (Savijoki et al. 2005). Following heat stress, 13 proteins were overexpressed, of which HtrA, DnaK and GroEL were also moderately induced by bile salts, indicating a close relationship between the heat and bile salt responses in this species. The work indicated that, as a consequence of prolonged heat stress, HtrA underwent sequential modification and proteolysis and that this mechanism could also be employed by *B. longum* to respond to heat stress.

The effects of mild pressure treatments prior to exposure to lethal temperatures, such as occur during spray drying, on heat tolerance in *L. rhamnosus* isolate GG, have been evaluated (Ananta and Knorr 2004). Cells that were pressure pre-treated at 100 MPa at 37 °C for 10 min had higher survival rates than untreated cells when exposed to heat challenge at 60 °C. To gain more insights into the cellular mechanism of this pressure-induced heat tolerance, flow cytometric analysis was applied in combination with a functional dye LIVE/DEADR BacLight bacterial viability kit. Dot plot analysis showed that a lower degree of membrane damage was observed in pressure pre-treated cells following heat treatment at 60 °C for 3 min than in non-pressure-treated cells. Evaluation of heat inactivation kinetics of pressure-treated cells in the presence of chloramphenicol, a protein synthesis inhibitor, demonstrated the potential contribution of pressure-induced protein biosynthesis in the enhancement of bacterial heat tolerance.

The evidence of potential cross-protection to different stresses has also been shown in *Lactococcus lactis* (Hartke et al. 1997; O'Sullivan and Condon 1997) and *P. freudenreichii* (Leverrier et al. 2004). Exposure to heat, acid and UV-radiation led to over-expression of GroEL and GroES proteins although quantitative analysis of the 2-DE gels indicated that the strength of induction was dependent on the type of stress stimulus; GroES was 12-fold increased in expression to heat stress but only 3.8-fold in response to acid stress (pH 5.5). However, in *P. freudenreichii* cross-protection to heat stress following acid pretreatment did not occur and the

acid pretreatment sensitised the cells to bile-salt stress. This suggests that cross-protection is not reciprocal; bile salt adaptation led to protection against acid and heat but adaptation to acid did not lead to protection against bile salt or heat. Furthermore, this indicates that despite the fact that the stress proteins are highly conserved, their regulation may vary between organisms (Cohen 2007).

4.3.5 Cold Stress

During industrial processes, like frozen storage of starter cultures, low temperature fermentation during cheese ripening and refrigerated storage of fermented products, LAB are exposed to temperatures far below their optimal growth temperature (Van de Guchte et al. 2002). Cold shock alters the usual crystalline nature of cell membranes and changes them to a gel phase state; it affects DNA supercoiling and the stability of some of the mRNAs that encode for proteins involved in the cold shock response (Phadtare et al. 2000). A better understanding of the responses to low temperatures and freezing could help optimise fermentation processes, improve survival of LAB during freezing and low temperature storage and thereby enhance their industrial performance.

Cold pretreatment can improve the survival of *Lactococcus lactis* (Panoff et al. 1995; Kim and Dunn 1997; Broadbent and Lin 1999), *S. thermophilus* (Wouters et al. 1999a) and *E. faecalis* (Thammavongs et al. 1996) during freezing and freeze-thaw cycles. The cold shock treatment also resulted in an increase of 6–8 % in both viability and probiotic properties of *P. acidilactici*, *L. plantarum* and *L. salivarius* following lyophilisation (freeze-drying), compared with the same species that had not received prior cold shock treatment (Reddy et al. 2009). Recently, 51 isolates of *L. delbrueckii* subsp. *lactis* isolated from Swiss cheeses were tested for autolytic activity and survival rate following freezing or lyophilisation (Koch et al. 2008). All isolates tested, including highly autolytic reference strains, showed autolytic activities in the range of 15–40 %, which were lower than in the control isolates of *Propionibacterium* (83.76 %) and *Bacillus* species (75 %). All 51 isolates survived freezing well (higher than 50 % survival), whereas resistance to lyophilisation was in the range of 0.49–63 % and isolate-dependent. There was no correlation between autolytic activity and survival after freezing or lyophilisation for the isolates tested.

The most strongly over-expressed proteins following cold treatment include a family of closely related low-molecular weight (~7 kDa) proteins termed cold shock proteins (Csp). These proteins share a high degree of sequence identity (>45 %) and orthologues have been found in multiple copies (from 2 to 9) in many Gram-positive and Gram-negative bacteria (Wouters et al. 2000a). The number of Csps present varied depending on isolate; for example one and six Csp proteins were reported from *S. thermophilus* isolate PB18 (Perrin et al. 1999) and isolate CNRZ302 (Wouters et al. 2000a), respectively. Similarly in *Lactococcus lactis*, the complete genome sequence of isolate IL1403, a *Lactococcus lactis* subsp.

lactis isolate revealed only two Csps (Bolotin et al. 2001) although genetic and 2-DE analysis revealed seven Csps in *Lactococcus lactis* subsp. *cremoris* isolate MG1363 (Wouters et al. 2000b). Among the CSPs in *Lactococcus lactis*, over-production of CspD at 30 °C resulted in a 2–10-fold increase in survival after freezing compared to control cells (Wouters et al. 1999b). Through transcriptional analysis, at least five Csp proteins, CspA, CspB, CspC, CspD and CspF were involved in the cold shock response in *Lactococcus Lactis* (Wouters et al. 1998).

Proteomic analysis of a ptsH3 mutant of *L. casei* found that the cold shock protein, CspA, was significantly overexpressed compared to the wild-type strain (Beaufils et al. 2007). They also noticed that CspA and CspB in *L. casei*, and a range of CSPs in other organisms exhibited significant sequence similarity at the C-terminal end of EIIA (Glc), a glucose-specific component of the phosphoenol-pyruvate: sugar phosphotransferase system. This similarity suggested a direct interaction between HPr and CSPs, as histidyl-phosphorylated HPr has been shown to phosphorylate EIIA (Glc) at its C-terminal end. This finding led to further studies comparing the cold shock responses of several carbon catabolite repression mutants to wild-type isolates. Following a shift from 37 °C to lower temperatures (20, 15 or 10 °C), all mutants showed significantly reduced growth rates (Monedero et al. 2007). Moreover, glucose-grown mutants that are unable to form P-Ser-HPr (ptsH1, hprK) exhibited drastically increased sensitivity to freeze/thaw cycles. However, when the same mutants were grown on ribose or maltose, their response to freezing and thawing was the same as the wild-type isolates (Perrin et al. 1999). Although subsequent biochemical and genetic studies were not able to identify the form of HPr implicated in tolerance to cold and freezing conditions, they strongly suggested a direct interaction between HPr or one of its phospho-derivatives and CspA and/or another, hitherto undetected cold shock protein in *L. casei*. Monedero et al. (2007) also verified, using a proteomics approach, the connection between the phosphotransferase system of *L. casei* and cold shock responses.

Interestingly, a major 7 kDa Csp protein was identified in the psychrotrophic biopreservative *Lactococcus piscium* isolate CNCM I-4031, but growth behaviour and proteomic responses were not cold regulated following cold shock or during cold acclimation (Garnier et al. 2010). In addition to other CSPs, proteins involved in oxidative stress responses, and fatty acid and energetic metabolism were upregulated following cold stress in *Lactococcus piscium*.

Besides the stress protein, ATP-dependent ClpP, two cold-induced proteins: pyruvate kinase and a putative glycoprotein endopeptidase were found over-expressed in *L. acidophilus* isolate RD758 during cold adaptation. An increase in the unsaturated to saturated fatty acid ratio and in the relative cycC19:0 fatty acid concentration was also observed in response to cold shock (Wang et al. 2005).

Cross-protection phenomena have also been detected in *L. delbrueckii* subsp. *bulgaricus* isolate CFL1 (Streit et al. 2008). When cells of this isolate were challenged with acid at the end of the fermentation process (pH 5.25 for 30 min) their cryotolerance was improved compared with cells grown under standard conditions (pH 6.0). Analysis of the cytosolic proteome established that changes occurred in the synthesis of 21 proteins involved in energy metabolism, nucleotide

and protein synthesis, and stress responses. Acid challenge also induced a slight decrease in the unsaturated to saturated and cyclic to saturated fatty acid ratios in cell membranes.

In a proteome study of *L. sanfranciscensis* the range of proteins induced by challenges by either high hydrostatic pressure (HHP), cold shock or NaCl stress were extremely similar (Hörmann et al. 2006). Interestingly, in this study of high pressure responses in *L. sanfranciscensis*, two differentially expressed proteins were identified by LC-MS as putative homologues to the cold shock proteins of *Lactococcus lactis* (Drews et al. 2002). All these findings may be useful for improving cold tolerance in LAB, either in cell banks or during industrial processing.

4.3.6 Salt Stress

During industrial processing, LAB are often exposed to salt. In China, several fermented foods produced using LAB as a starter culture also use salt to reduce water activity, e.g. in the production of *suancai*, *labaicai*, *dajiang* and sichuan pickle. In general, a sudden increase in the osmolarity of the environment results in movement of water from bacterial cells into the external medium, resulting in turgor pressure loss, intracellular solute concentration changes and cell volume changes (Van Der Heide and Poolman 2000). Bacteria are able to counteract hyperosmotic stress by accumulating or synthesising compatible osmoprotectants at high intracellular concentrations without affecting vital cellular processes. In *Lactococcus lactis*, *L. plantarum, L. salivarius* and *B. breve*, the principal osmoprotectants are glycine-betaine and proline (Molenaar et al. 1993; Glaasker et al. 1996; Faiza et al. 2011; Sheehan et al. 2006; Sheehan et al. 2007). The operons *busA* and *opuA* encode for uptake systems in *Lactococcus lactis* and have a high affinity for glycine-betaine and proline (Obis et al. 1999; Obis et al. 2001; Bouvier et al. 2000; Xie et al. 2004). In contrast, proline, glycine-betaine and related molecules do not protect *O. oeni* during osmotic stress (Le Marrec et al. 2007). Instead, studies suggest that proline- and glutamate-containing peptides contribute to the adaptation of *O. oeni* to high salt through their intracellular hydrolysis and/or direct accumulation. Proline- and glutamate-containing peptides are also implicated as osmoprotectants in *L. zeae* where the addition of di- and tri-peptides individually increased osmotolerance in this species when grown in a CDM containing NaCl (Piuri et al. 2003).

Cell walls and membranes are the first line of defence in Gram-positive bacteria including LAB and play a role in salt tolerance. In *L. bulgaricus*, it was revealed that the lipid composition of membranes changed during growth in high salt environments (Tymczyszyn et al. 2005). This change in lipid composition is also likely to be linked to osmotic activation of the osmoprotectant transporter system in *Lactococcus lactis* and *L. casei* (Piuri et al. 2005).

Significant advances have been made towards achieving a global picture of the osmotic stress response of LAB at a genomic and cellular scale by analysing the

proteome during salt stress (Tsakalidou and Papadimitriou 2011). Using 2-DE, ten out of 52 overexpressed proteins in *S. mutans* and 12 out of 96 in *E. faecalis* were specifically induced by salt stress (Svensater et al. 2000; Pichereau et al. 1999). In *Lactococcus lactis* isolate MG1363, an overlap between heat and salt stress responses has been reported, demonstrating that all salt stress-induced proteins were also induced by heat stress (Kilstrup et al. 1997). Hsp 60, Hsp 70 and a fatty acid biosynthesis enemy (Fab G) are thought to play an important role in *L. paracasei* isolate LN-1 tolerance to salt stress (Wu et al. unpublished data). Marceau et al. (2004) observed significant variation in a set of 21 proteins produced by *L. sakei* cells grown either at 4 °C or in the presence of 4 % NaCl (Marceau et al. 2004). Comparative proteomic analyses of *Lactococcus lactis* subsp. *cremoris* isolate SK11 cells that either contained or did not contain glutathione and were then exposed or not exposed to osmotic stress have recently been made (Zhang et al. 2010b). The results revealed that 21 of 29 differentially expressed proteins were involved in metabolic pathways, mainly sugar metabolism.

4.3.7 Nutrient Supplement

4.3.7.1 LAB Cultured in Medium

Early studies by a number of respected researchers have established that LAB require complex media for rapid growth (e.g. Sneath et al. (1986)). In the laboratory, synthetic media, such as MRS and M17, have been used for many years to culture LAB. Among the constituents of such media, sugar is the most important for these organisms. Sugar catabolism is crucial for the generation of energy and biomass, but also for the conversion of carbohydrates to lactate which is most important for the fermentation process employed within the food technology industry (Pessione et al. 2010). Also, the type of sugar fermentation is a key attribute for identification of LAB species.

In LAB glucose and lactose are the most important and wildly utilised sugars. The rapid growth of *Lactococcus* species in milk leads to efficient conversion of lactose into lactic acid (homofermentation). The resulting acidification of the medium causes protein coagulation, a property that is exploited in the manufacture of cheese. Even et al. (2001) not only established the reference map for *Lactococcus lactis*, but also compared glucose- and lactose-grown cells at the proteome level (Marceau et al. 2004). When rapidly growing on glucose in M17 media, the homofermentative metabolism of *Lactococcus lactis* led to the dissimilation of 90 % of the sugar into lactate (Marceau et al. 2004). The enzyme was identified as a major spot on the 2-DE maps. They also found that growth of *Lactococcus lactis* on lactose was associated with a decrease in pyrimidine-regulated enzymes.

To elucidate the fructose metabolism pathway in *B. longum* isolate NCC2705 and construct a fermentation model, Sun et al. (2008) compared the proteome of this isolate when it was cultivated either on glucose or fructose. MALDI-TOF MS

and ESI-MS/MS were used to identify the differentially expressed proteins (Sun et al. 2008). With semi-quantitative RT-PCR the study quantified distinctively expressed proteins at the level of transcription. Proteomic comparison of glucose- and fructose-grown cells demonstrated much similarity. All the enzymes and proteins that were present during glucose degradation were also there during fructose degradation. However, using MALDI-TOF/MS, there was a more than three-fold difference in expression level for nine identified spots representing five protein entries. The sugar-binding protein specific to fructose (BL0033) and an ABC transporter ATP-binding protein (BL0034) showed higher expression levels in cells grown on fructose than on glucose. This was confirmed by semi-quantitative RT-PCR. Time course and concentration experiments with BL0033 showed that the induction time was directly correlated with high fructose concentrations. Fructose was catabolised via the same degradation pathway as glucose at the proteomics level and BL0033 was induced by fructose. All results suggested that the uptake of fructose into the cell was conducted by a specific ABC transporter system, in which BL0033 and BL0034 were likely to play an important role.

Moreover, as probiotics, research on the growth of species in the genus *Bifidobacterium* has attracted a lot of attention. Recent studies have demonstrated that XOS, which are classified as emerging prebiotics, selectively enhanced the growth of *B. animalis* subsp. *lactis* isolates. XOS are sugar oligomers composed of α-1, 4-linked xylopyranosyl backbone which are obtained by either chemical or, more commonly, enzymatic hydrolysis of xylan polysaccharides extracted from plant cell walls. The putative prebiotic effects of XOS were demonstrated both by in vitro studies (Rycroft et al. 2001) and by small-scale in vivo studies in humans (Chung et al. 2007). A few XOS-degrading enzymes have been identified and characterised in the genus *Bifidobacterium*. Specifically, a *β*-D-xylosidase characterised from *B. breve* isolate K-110 was shown to hydrolyse xylan to xylose (Shin et al. 2003). To elucidate the metabolism of XOS in the well documented and widely used probiotic isolate of *B. animalis* subsp. *lactis*, BB-12, a combined proteomic and transcriptomic approach was used involving DNA microarrays, qPCR and 2D-DIGE analyses of samples from cultures grown on either XOS or glucose (Gilad et al. 2010). The analyses showed that nine of the ten genes that encoded proteins predicted to play a role in XOS catabolism (i.e. XOS-degrading and -metabolising enzymes, transport proteins and a regulatory protein) were induced by XOS at the transcriptional level and the proteins encoded by three of these (*β*-D-xylosidase, sugar-binding protein and xylose isomerase) were produced at higher levels on XOS. Based on these results a model for the catabolism of XOS in isolate BB-12 was proposed as follows: isolate BB-12 utilises an ATP-binding cassette (ABC) transport system (probably for oligosaccharides) to bind XOS to the cell wall surface and to transport them into the cell; XOS are then degraded intracellularly by xylanases and xylosidases to D-xylose, which is subsequently metabolised by the D-fructose-6-P shunt. The findings obtained in this study may have implications for the design of a symbiotic application containing BB-12 and XOS.

Another study focused on the enhancing effect of the prebiotics, *β*-glucans, on growth of *B. longum* subsp. *infantis*. A comparative proteomic analysis was done

including 2D-DIGE, qRT-PCR and enzyme activity assays on samples obtained from cultures grown on *β*-glucans derived either from barley, seaweed or mushroom (Zhao and Cheung 2013). Results showed that 77 spots were differentially expressed on the different culture media and 17 of them were predicted to play a role in *β*-glucan catabolism, including the ABC transporter for sugars, enolase and the phosphotransferase system protein. Amongst these, six genes encoding for six proteins were shown to be over-expressed by *β*-glucans at the transcriptional level and were found at higher levels. An enzyme activity assay detected intracellular glucanase activity in samples from cultures grown on the *β*-glucans from seaweed and mushroom. On the basis of these results, a model for catabolism of *β*-glucans in *B. infantis* was proposed as follows: *β*-glucan molecules in the medium are transported into the cell through the ABC transport system and the PTS (phosphotransferase system); this is followed by hydrolysis by intracellular glucanase to glucose which is subsequently incorporated into the central fermentative pathway 'bifid shunt'. This study revealed, for the first time, the possible degradation pathway of *β*-glucans by *B. infantis*, which has implications for the potential use of *β*-glucans as novel prebiotics in the development of symbiotic applications.

4.3.7.2 LAB Cultured in Milk and Soybean Milk

In order to elucidate the metabolic processes involved in growth of LAB in an industrial environment, proteome analyses have been used on *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus* and *Lactococcus lactis* cultivated in milk (Rechinger et al. 2000; Derzelle et al. 2005; Gitton et al. 2005).

Using 2-DE analysis to compare protein synthesis by *L. delbrueckii* ssp. *bulgaricus* isolate NCFB 2772 during the first 30 min of fermentation in either milk or standard MRS media revealed that, regardless of whether the carbon source was glucose (MRS) or lactose (milk), there were only low levels of synthesis of stress or glycolytic enzymes (Rechinger et al. 2000). However, proteomic comparison between this isolate and *S. thermophilus* isolate LMG18311 growing either in the synthetic medium M17 or skimmed milk showed that 55 proteins were expressed differently in the two media by both species (Derzelle et al. 2005). Strong over-expression of the pyruvate formatelyase (PFL) supplied formate for anabolic purposes.

Herve-Jimenez et al. (2008) analysed the physiology of *S. thermophilus* isolate LMG18311 during the late stationary phase of grown in milk (between 2h30 and 5h30) using proteomic and transcriptomic approaches (Herve-Jimenez et al. 2008). The 2-DE map comprised 203 identified proteins corresponding to 32 % of the theoretical proteome. It revealed the over-expression of peptides, AA transporters (especially for sulfur AA biosynthesis) and genes and proteins involved in the metabolism of various sugars.

Subsequently the same research group studied *S. thermophilus* cultured as a mixture in milk with *L. delbrueckii* subsp. *bulgaricus* by global monitoring of transcription and protein abundance during two growth stages (Herve-Jimenez

et al. 2009). As is well known, *L. delbrueckii* subsp. *bulgaricus* is able to supply peptides and AA for *S. thermophilus* and, in turn, *S. thermophilus* can produce formic acid and carbon dioxide to stimulate the growth of *L. delbrueckii* subsp. *bulgaricus*. However, this study first supplied the data from gene and protein expression to support the above point of view. The study revealed a 4.1 % alteration in the *S. thermophilus* transcriptome and proteome, triggering modifications in specific aspects of cellular metabolism, including the over-expression of BCAA and Arg biosynthesis, the upregulation of several genes involved in nucleic acid metabolism (*polC* and *ssbB*) and in translation (ribosomal proteins, tRNA synthetases and *gatB* (a subunit of the Glu-tRNA^{Gln} amidotransferase), which may be connected with the better growth of *S. thermophilus* in the mixed culture than in the monoculture grew. Moreover, the authors suggested that *L. delbrueckii* subsp. *bulgaricus* was providing purines or their precursors to *S. thermophilus* that corresponding to the downregulation of purine biosynthesis pathway in *S. thermophilus*.

Further, Sieuwerts et al. (2010) reported the application of mixed culture transcriptome profiling and a systematic analysis of the effect of interaction-related compounds on growth, which allowed them to unravel the molecular responses associated with batch mixed culture growth in milk of *S. thermophilus* isolate CNRZ1066 and *L. bulgaricus* isolate ATCC BAA-365 (Sieuwerts et al. 2010). The study confirmed that the interactions between these bacteria were primarily related to purine, amino acid and long-chain fatty acid metabolism.

Our own laboratory study of the fermentation properties of the probiotic LAB *L. casei* Zhang isolate indicated that it grew faster in soymilk than in bovine milk at inoculation rates between 2×10^6 and 2×10^7 cfu g^{-1} (Wang et al. 2008). Soymilk has received increasing attention recently as it is now used for the production of many yoghurt-like products. Proteomic analysis has been done to determine whether the different growth conditions provided by milk and soymilk resulted in differential expression of proteins in *L. casei* Zhang isolate (Wang et al. 2013). Functional analysis revealed that the majority of proteins identified were associated with the transport and metabolism of carbohydrates, nucleotides and amino acids. These results were confirmed by qRT-PCR assays which showed that the loci involved in purine and pyrimidine biosynthesis were transcriptionally enhanced during growth in soymilk during the lag phase (pH 6.4) but not in bovine milk, whereas the loci involved in carbohydrate metabolism were upregulated in bovine milk and not soymilk. In previous, *Lactococcus lactis* isolate NCDO763 synthesised 11 enzymes that were directly involved in the de novo synthesis of purine bases at high levels cultivated in milk than chemical medium (Gitton et al. 2005). Thus, we suggest that, like *Lactococcus lactis*, the expressions of enzymes that are involved in de novo synthesis of purine are also important for the growth of *L. casei* in both milk and soymilk. The higher expression of proteins related to purine and pyrimidine metabolism in *L. casei* during growth in soymilk than in milk at lag phase may be connected with the faster growth rate in soymilk. Furthermore, our results also showed that L-glutamine may play an important role in the growth of *L. casei* in both soymilk and bovine milk perhaps by contributing to purine,

pyrimidine, and aminosugar metabolism. Recently, we have published two similar studies regarding the transcriptome analysis of *L. casei* Zhang isolate during fermentation in milk and soymilk (Wang et al. 2011a, b). However, because data were analysed differently they cannot be correlated with the previous study. Further interpretation of these two different data sets will be addressed in future work.

Recently, metabolic and proteomic adaptation in *L. rhamnosus* isolates from different stages of ripening Parmigiano Reggiano cheese have been reported when grown in different media (Bove et al. 2012). Compared to MRS broth, cultivation under cheese-like conditions (cheese broth, CB) increased the number of free amino acids used as carbon sources. Compared with growth on MRS or pasteurised and microfiltered milk, all isolates cultured in CB had low levels of synthesis of d,l-lactic acid and elevated levels of acetic acid. The proteomic maps of the five representative isolates, showing different metabolic traits, were compared after growth on either MRS or CB media. Protein spots which under-expressed (62 spots) or over-expressed (59 spot) in intensity during growth on CB were identified using MALDI-TOF-MS/MS or LC-nano-ESI-MS/MS. Compared with cultivation on MRS broth, the *L. rhamnosus* isolates cultivated on CB had different quantities of some proteins responsible for protein biosynthesis, nucleotide and carbohydrate metabolisms, the glycolysis pathway, proteolytic activity, cell wall and exopolysaccharide biosynthesis, cell regulation, amino acid and citrate metabolism, oxidation/reduction processes and stress responses. Further, the quantity of intracellular and cell-associated proteins was not only affected by culture conditions but also varied depending on the isolate and the stage of ripening the cheese had achieved when the bacteria were isolated. It is, therefore, important to compare proteome patterns amongst isolates of the same species.

4.4 Comparison of LAB Proteomes Amongst Isolates of the Same Species

There are strict criteria for selection of probiotic bacteria, particularly related to their ability to survive during passage through the acidic conditions of the stomach and the bile salts in the upper intestine in sufficient numbers to influence the colon microenvironment (Fuller 1989). Because probiotic properties are isolate-dependent, the probiotic potential of different bacterial isolates, even within the same species, differs (Isolauri et al. 2004). In conjunction with the development of genomics, proteomics and bioinformatics recently, more information on LAB allows identification of unique targets for isolate characterisation and differentiation.

Makarova et al. (2007) has reported on the comparative genomics of LAB and its application to evolutionary reconstruction and functional genomics (Makarova et al. 2007). To further understand these kinds of differences in physiological characteristics amongst isolates within species or subspecies, proteome analysis of global detectable protein expression patterns are appropriate and useful.

4.4.1 Comparisons of Metabolism

Under the same medium culture environment, the overall protein expression pattern of the isolates within the same specie is similar. Using 2-DE maps our research group compared two isolates of *Lb casei*, the Zhang isolate and isolate XM2-1, both from traditionally fermented koumiss in Inner Mongolia (Wu et al., unpublished data). As described previously, the Zhang isolate showed all the characteristics considered necessary as a probiotic bacterium, especially higher tolerance to acidic conditions than other *L. casei* isolates including XM2-1. Based on analysis of the expression patterns of the two *L. casei* isolates, the numbers of protein spots quantified from the gels run at a pH range from 4 to 7 were around 606 ± 08 (Zhang) and 610 ± 10 (XM2-1), respectively. Although the overall expression patterns of proteins for the two isolates were similar, 44 protein spots showing statistically significant differences in levels of expression, including pyruvate kinase, L-2-lactate dehydrogenase, N-acetylglucosamine-6-phosphate deacetylase, S-ribosylhomocysteinase, the proteolytic subunit of an ATP-dependent Clp protease, EF-Tu, EF-Ts and 30S ribosomal proteins S2. Among the differences, fructose bisphosphate aldolase, a regulatory glycolytic enzyme that had different molecular weights on the proteomic maps of the two isolates, may be an important factor causing the variability in tolerance to acid which needed further working.

Recently, Aires et al. (2010) compared three human-derived *B. longum* isolates with the sequenced model isolate *B. longum* isolate NCC2705 at the chromosome and proteome levels (Aires et al. 2010). Based on 2-DE analysis, only 45 different expressed proteins among all the isolates were found. Except for some belonging to the carbohydrate metabolism, most of the different expressed proteins were identified as cell wall or cell membrane synthesis proteins. Similar results were also reported by Savijoki et al. (2011). The study compared the proteome analysis of two *L. rhamnosus* isolates, the well-known probiotic isolate GG and the dairy isolate Lc705 using 1-DE and LC-MS/MS (Savijoki et al. 2011). More than 90 proteins in GG and 150 in Lc705 were found to be differently expressed, and some of the proteins were related to the cell wall function and immunomodulation which is worth noting.

4.4.2 Differences in Stress Responses

Stress response analysis of different strains belonging to the same specie using proteome techniques can not only be valuable for investigating the common differences for better explanation the tolerance mechanism involved in LAB, but can also provide a better understanding of intraspecies diversity of LAB and their potential use as probiotics.

4.4.2.1 Bile and Heat Stress Response

Recently, Hamon et al. (2011) investigated the natural protein diversity within the *L. plantarum* species in relation to bile tolerance using comparative proteomics (Hamon et al. 2011). Bile tolerance properties of nine *L. plantarum* isolates were studied in vitro. Three with different bile tolerance levels (*L. plantarum* isolate 299 V [tolerant], *L. plantarum* isolate LC 804 [intermediate] and *L. plantarum* isolate LC 56 [sensitive]), were selected for further proteome analysis using 2-DE, tryptic digestion, LC-MS. The study revealed six proteins that may be key in the bile salt response and adaptation in *L. plantarum*: two glutathione reductases involved in protection against oxidative injury caused by bile salts, a cyclopropane-fatty-acyl-phospholipid synthase implicated in maintenance of cell envelope integrity, a bile salt hydrolase, an ABC transporter and a F_0F_1-ATP synthase that participated in the active removal of bile-related stress factors. The study can be the example showing that the comparative proteomic analysis can help understand the differential bacterial properties of LAB.

Besides, differential protein expression in two *B. longum* isolates that differed in their heat shock tolerance has been described using a comparative qualitative survey of their proteomes and relative LC-MS/MS-based label-free protein quantification (Guillaume et al. 2009). Among the differential expressed proteins induced by heat, 19 proteins were common to both isolates, including GroEL, ClpA/B, ClpC, GrpE and DnaK, which were regarded as general stress response proteins.

4.4.2.2 Nutrition Stress Response

Proteomic analysis can also be used to study sugar utilisation difference between isolates within same specie. McLeod et al. (2010) compared primary metabolism in ten isolates of *L. sakei* from meat and fish after growth on media with either glucose or ribose as the main sugar available (McLeod et al. 2010). There were similarity protein pattern among isolates, and in total, ten proteins were upregulated in all or most of the strains after growth on ribose. Among the induced proteins, RbsD, the D-ribose pyranase, RbsK, the ribokinase and Xpk, the putative phosphoketolase which involved in ribose catabolism were identified. Moreover, expression of enzymes involved in pyruvate and glycerol/glycerolipid metabolism were also affected by the change of carbon source.

In addition, a comparative proteome study of two isolates of *L. plantarum* (REB1 and MLBPL1) grown in commercial medium (MRS broth), cucumber juice or liquid pig feed were performed to explore changes in the metabolic pathways of these bacteria using 2-DE (Plumed-Ferrer et al. 2008). Bacterial growth was significantly greater in cucumber juice and liquid pig feed than in MRS broth due to the malate present in cucumber juice and liquid pig feed. Although there was similarity between two strains, REB1 had more proteins that were strongly up-/downregulated than those of MLBPL1. From the map comparison, 80 and 55

protein spots from REB1 and MLBPL1 showed statistically significant differences between media, respectively. Further analysis indicated that the highest expression proteins in MRS broth were belonging to glycolysis and pyruvate metabolism. In liquid feed, glycolytic enzymes and those involved in pyruvate metabolism generally showed the lowest expression. This study has improved our understanding of the mechanisms involved in the growth performance of LAB isolates to be used for food and feed fermentation, information that is of crucial importance in obtaining a high-quality fermented product.

4.4.3 Differences in Mixed Culture Compared With Monoculture

Recently, the influence of mixed culture of *B. longum* isolate NCIMB8809 and *B. breve* isolate NCIMB8807 on their physiology has been studied (Ruiz et al. 2009b). Just as mentioned before, in the dairy industry, some species in the genus *Bifidobacterium* are probiotic and commonly added to functional food products (Fouhy et al. 2013). In this study, 2-DE separation of protein extracts, coupled with MS protein analysis allowed the identification of 16 proteins whose expression drastically changed when cells were grown in compartmentalised mixed culture compared with monoculture. These included ribosomal proteins and proteins involved in carbohydrate metabolism, gene regulation, cell envelope biogenesis and transport processes. Significant changes in some glycoside-hydrolysing activities (β-D-xylopyranosidase, α-Larabinofuranosidase and β-D-glucopyranosidase) were also detected. Furthermore, qRT-PCR experiments using targets in the *B. breve klgR* (transcriptional regulator) *clpP1*, *clpP2* and *clp*C (chaperone- and protease-encoding genes positively regulated by *clgR*) genes supported the proteomic results; the four genes displayed a higher expression level in mixture culture. This study provided new insights into the understanding of communication between *Bifidobacterium* species.

4.5 Conclusion

In proteomics, the researches have focused on the whole proteins of LAB and the response to environmental stress or growth condition. A comprehensive description of stress proteins of LAB and analysis of their expression pattern under different environmental conditions would greatly increase our understanding of the molecular mechanisms underlying the extraordinary capacity of the bacteria to survive under hostile conditions. Further, understanding the metabolic mechanisms underlying the growth performance of a LAB isolate to be used for food fermentation is necessary to achieve the highest quality and safest products.

In molecular biology, the omics includes genomics, proteomics, metabolomics, transcriptomics, lipidlipidomics, immunomics, glycomics and RNomics, etc. As largest source for probiotics, the omics study of LAB is getting significance attention. The identification of cell components involved in LAB activities is a challenge in current microbiota research. It is worth noting that some complete studies have combined proteomics, genomics, transcriptomic and metabolomics together in order to understand the response of LAB under stress better. This may have become a trend in recent research. Therefore, much more detailed and extensive knowledge is needed for researchers to study and utillise LAB. Meanwhile, the advancement of technology will probably promote the scientific development. Thus new techniques in proteomics are still urgently needed.

References

Accolas JP, Blocques R, Didiene R, Regnier J. Propriétés acidifiantes des bactéries lactiques thermophiles en relation avec la fabrication du yoghourt. Le Lait. 1977;57:1–23.

Ahrné S, Molin G, Nobaek S, Jeppsson B, Adlerberth I, Wold AE. The normal lactobacillus flora of healthy human rectal and oral mucosa. J Appl Microbiol. 1998;85:88–94.

Aires J, Anglade P, Baraige F, Zagorec M, Champomier-Vergès MC, Butel MJ. Proteomic comparison of the cytosolic proteins of three *Bifidobacterium longum* human isolates and *B. longum* NCC2705. BMC Microbiol. 2010;10:29.

Ananta E, Knorr D. Evidence on the role of protein biosynthesis in the induction of heat tolerance of *Lactobacillus rhamnosus* GG by pressure pre-treatment. Int J Food Microbiol. 2004;96:307–13.

Anderson NG, Anderson NL. Twenty years of two-dimensional electrophoresis: Past, present and future. Electrophoresis. 1996;17:443–53.

Anglade P, Demey E, Labas V, Le Caer JP, Chich JF. Towards a proteomic map of *Lactococcus lactis* NCDO 763. Electrophoresis. 2000;21:2546–9.

Arena S, D'Ambrosio C, Renzone G, Rullo R, Ledda L, Vitale F, Maglione G, Varcamonti M, Ferrara L, Scaloni A. A study of *Streptococcus thermophilus* proteome by integrated analytical procedures and differential expression investigations. Proteomics. 2006;6:181–92.

Banerjee S, Mazumdar S. Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. Int J Anal Chem. 2012;2012:1–40.

Beaufils S, Sauvageot N, Mazé A, Laplace JM, Auffray Y, Deutscher J, Hartke A. The cold shock response of *Lactobacillus casei*: relation between HPr phosphorylation and resistance to freeze/thaw cycles. J Mol Microbiol Biotechnol. 2007;13:65–75.

Begley M, Gahan CGM, Hill C. Bile stress response in *Listeria monocytogenes* LO28: adaptation, crossprotection and identification of genetic loci involved in bile resistance. Appl Environ Microbiol. 2002;68:6005–12.

Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. FEMS Microbiol. 2005;29:625–51.

Bessarabova M, Ishkin A, JeBailey L, Nikolskaya T, Nikolsky Y. Knowledge-based analysis of proteomics data. BMC Bioinformatics. 2012;13:S13.

Bezkorovainy A. Probiotics: determinants of survival and growth in the gut. Am J Clin Nutr. 2001;73:399–405.

Blackstock WP, Weir MP. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol. 1999;17:121–7.

Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, Ehrlich SD, Sorokin A. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. 2001;11:731–53.

Bossi A, Rinalducci S, Zolla L, Antonioli P, Righetti PG, Zapparoli G. Effect of tannic acid on *Lactobacillus hilgardii* analysed by a proteomic approach. J Appl Microbiol. 2007;102:787–95.

Bouvier J, Bordes P, Romeo Y, Fourçans A, Bouvier I, Gutierrez C. Characterization of OpuA, a Glycine-Betaine uptake system of *Lactococcus lactis*. J Mol Microbiol Biotechnol. 2000;2:199–205.

Bove CG, De Angelis M, Gatti M, Calasso M, Neviani E, Gobbetti M. Metabolic and proteomic adaptation of *Lactobacillus rhamnosus* strains during growth under cheese-like environmental conditions compared to de man, rogosa, and sharpe medium. Proteomics. 2012;12:3206–18.

Boyle RJ, Bath-Hextall FJ, Leonardi-Bee J, Murrell DF, Tang ML. Probiotics for the treatment of eczema: a systematic review. Clin Exp Allergy. 2009;39:1117–27.

Broadbent JR, Lin C. Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. Cryobiol. 1999;39:88–102.

Broadbent JR, Oberg JC, Wang H, Wei L. Attributes of the heat shock response in three species of dairy *Lactobacillus*. Syst Appl Microbiol. 1997;20:12–9.

Bron PA. The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Thesis of Wageningen University, Netherlands; 2003. p. 90–108.

Budin-Verneuil A, Pichereau V, Auffray Y, Ehrlich DS, Maguin E. Proteomic characterization of the acid tolerance response in *Lactococcus lactis* MG1363. Proteomics. 2005;5:4794–807.

Burns P, Sánchez B, Vinderola G, Ruas-Madiedo P, Ruiz L, Margolles A, Reinheimer J, de los Reyes-Gavilan CG. Inside the adaptation process of Lactobacillus delbrueckii subsp. lactis to bile. Int J Food Microbiol. 2010;142:132–41.

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. Blue silver: a very sensitive colloidal coomassie G-250 staining for proteome analysis. Electrophoresis. 2004;25:1327–33.

Champomier-Vergès MC, Maguin E, Mistou MY, Anglade P, Chich JF. Lactic acid bacteria and proteomics: current knowledge and perspectives. J Chromatogr B Analyt Technol Biomed Life Sci. 2002;771:329–42.

Chung Y, Hsu C, Ko C, Chan Y. Dietary intake of xylooligosaccharides improves the intestinal microbiota, fecal moisture, and pH value in the elderly. Nutr Res. 2007;27:756–61.

Clauser KR, Baker P, Burlingame AL. Role of accurate mass measurement (±10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal Chem. 1999;71:2871–82.

Coenye T, Vandamme P. Extracting phylogenetic information from whole-genome sequencing projects: the lactic acid bacteria as a test case. Microbiol. 2003;149:3507–17.

Coeuret V, Gueguen M, Vernoux JP. Numbers and strains of lactobacilli in some probiotic products. Int J Food Microbiol. 2004;97:147–56.

Cohen DPA. Functional analysis of *Lactobacillus plantarum* WCFS1: a proteomic approach. PhD. thesis Wageningen University, Wageningen, The Netherlands, with summary in Dutch; 2007. p. 32–7.

Cohen DP, Renes J, Bouwman FG, Zoetendal EG, Mariman E, de Vos WM, Vaughan EE. Proteomic analysis of log to stationary growth phase *Lactobacillus plantarum* cells and a 2-DE database. Proteomics. 2006;6:6485–93.

Colinge J, Bennett KL. Introduction to computational proteomics. PLoS Comput Biol. 2007;3:e114.

Corthals GL, Wasinger VC, Hochstrasser DF, Sánchez JC. The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis. 2000;21:1104–15.

Curiel JA, Rodríguez H, de las Rivas B, Anglade P, Baraige F, Zagorec M, Champomier-Vergès M, Muñoz R, de Felipe FL. Response of a *Lactobacillus plantarum* human isolate to tannic acid challenge assessed by proteomic analyses. Mol Nutr Food Res. 2011;55:1454–65.

Curreem SO, Watt RM, Lau SK, Woo PC. Two-dimensional gel electrophoresis in bacterial proteomics. Protein Cell. 2012;3:346–63.

De Angelis M, Gobbetti M. A review: environmental stress responses in *Lactobacillus*. Proteomics. 2004;4:106–22.

De Angelis M, De Angelis M, Gobbetti M, Bini L, Pallini V, Cocconcelli PS. The acid-stress response in *Lactobacillus sanfranciscensis* CB1. Microbiol. 2001;147:1863–73.

De Angelis M, Di Cagno R, Huet C, Crecchio C, Fox PF, Gobbetti M. Heat shock response in *Lactobacillus plantarum*. Appl Environ Microbiol. 2004;70:1336–46.

De Boever P, Wouters R, Verschaeve L, Berckmans P, Schoeters G, Verstraete W. Protective effect of the bile salt hydrolase-active *Lactobacillus reuteri* against bile salt cytotoxicity. Appl Microbiol Biotechnol. 2000;53:709–14.

Derzelle S, Bolotin A, Mistou MY, Rul F. Proteome analysis of *Streptococcus thermophilus* grown in milk reveals pyruvate formate-lyase as the major upregulated protein. Appl Environ Microbiol. 2005;71:8597–605.

Desmond C, Stanton C, Fitzgerald GF, Collins K, Ross RP. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. Int Dairy J. 2001;11:801–8.

Desvaux M, Hébraud M, Talon R, Henderson IR. Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. Trends Microbiol. 2009;17:139–45.

Desvaux M, Dumas E, Chafsey I, Chambon C, Hébraud M. Comprehensive appraisal of the extracellular proteins from a monoderm bacterium: theoretical and empirical exoproteomes of *Listeria monocytogenes* EGD-e by secretomics. J Proteome Res. 2010;9:5076–92.

Di Cagno R, De Angelis M, Limitone A, Fox PF, Gobbetti M. Response of *Lactobacillus helveticus* PR4 to heat stress during propagation in cheese whey with a gradient of decreasing temperatures. Appl Environ Microbiol. 2006;72:4503–14.

Drews O, Weiss W, Reil G, Parlar H, Wait R, Görg A. High pressure effects step-wise altered protein expression in *Lactobacillus sanfranciscensis*. Proteomics. 2002;2:765–74.

Drews O, Reil G, Parlar H, Görg A. Setting up standards and a reference map for the alkaline proteome of the gram-positive bacterium *Lactococcus lactis*. Proteomics. 2004;4:1293–304.

Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney SM, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F, Collins JK. In vitro selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am J Clin Nutr. 2001;73:386–92.

Earnshaw RG, Appleyard J, Hurst RM. Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. Int J Food Microbiol. 1995;28:197–219.

Edelman S, Leskel S, Ron E, Apajalahti J, Korhonen TK. In vitro adhesion of an avian pathogenic *Escherichia coli* 078 strain to surfaces of the chicken intestinal tract and to ileal mucus. Vet Microbiol. 2003;91:41–56.

Even S, Lindley ND, Cocaign-Bousquet M. Molecular physiology of sugar catabolism in *Lactococcus lactis* IL1403. J Bacteriol. 2001;183:3817–24.

Faiza B, Halima Z, Nour-Eddine K. Physiological responses of salt stress and osmoprotection with proline in two strains of lactococci isolated from camel's milk in Southern algeria. Afr J Biotechnol. 2011;83:19429–35.

Flahaut S, Hartke A, Giard JC, Benachour A, Boutibonnes P, Auffray Y. Relationship between stress response toward bile salts, acid and heat treatment in *Enterococcus faecalis*. FEMS Microbiol Lett. 1996a;138:49–54.

Flahaut S, Frere J, Boutibonnes P, Auffray Y. Comparison of the bile salts and sodium dodecyl sulfate stress responses in *Enterococcus faecalis*. Appl Environ Microbiol. 1996b;62:2416–20.

Foster JW. Microbial responses to acid stress. Bacterial stress responses. In: Storz G, Hengge-Aronis R, editors. American society for microbiology press. DC: Washington; 2000. p. 99–116.

Foster JW, Hall HK. Inducible pH homeostasis and the acid tolerance response of Salmonella typhimurium. J Bacteriol. 1991;173:5129–35.

Fouhy F, O'Connell Motherway M, Fitzgerald GF, Ross RP, Stanton C, van Sinderen D, Cotter PD. In silico assigned resistance genes confer bifidobacterium with partial resistance to aminoglycosides but not to β-lactams. PLoS One. 2013;8:e82653.

Frees D, Savijoki K, Varmanen P, Ingmer H. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC gram-positive bacteria. Mol Microbiol. 2007;63:1285–95.

Fuller R. Probiotics in man and animals. J Appl Bacteriol. 1989;66:365–78.

Gardiner GE, O'Sullivan E, Kelly J, Auty MAE, Fitzgerald GF, Collins JK, Ross RP, Stanton C. Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. Appl Environ Microbiol. 2000;66:2605–12.

Garnier M, Matamoros S, Chevret D, Pilet MF, Leroi F, Tresse O. Adaptation to cold and proteomic responses of the psychrotrophic biopreservative *Lactococcus piscium* strain CNCM I-4031. Appl Environ Microbiol. 2010;76:8011–8.

Giard JC, Laplace JM, Rincé A, Pichereau V, Benachour A, Leboeuf C, Flahaut S, Auffray Y, Hartke A. The stress proteome of *Enterococcus faecalis*. Electrophoresis. 2001;22:2947–54.

Gilad O, Jacobsen S, Stuer-Lauridsen B, Pedersen MB, Garrigues C, Svensson B. Combined transcriptome and proteome analysis of *Bifidobacterium animalis* subsp. *lactis* BB-12 grown on xylo-oligosaccharides and a model of their utilization. Appl Environ Microbiol. 2010;76:7285–91.

Gilad O, Hjernø K, Sterlund EC, Margolles A, Svensson B, Stuer-Lauridsen B, Møller AL, Jacobsen S. Insights into physiological traits of *Bifidobacterium animalis* subsp. *lactis* BB-12 through membrane proteome analysis. Proteomics. 2011a;11:3935–41.

Gilad O, Svensson B, Viborg AH, Stuer-Lauridsen B, Jacobsen S. The extracellular proteome of *Bifidobacterium animalis* subsp. *lactis* BB-12 reveals proteins with putative roles in probiotic effects. Proteomics. 2011b;11:2503–14.

Gitton C, Meyrand M, Wang J, Caron C, Trubuil A, Guillot A, Mistou MY. Proteomic signature of *Lactococcus lactis* NCDO763 cultivated in milk. Appl Environ Microbiol. 2005;71:7152–63.

Glaasker E, Konings WN, Poolman B. Osmotic regulation of intracellular solute pools in *Lactobacillus plantarum*. J Bacteriol. 1996;178:575–82.

González-Márquez H, Perrin C, Bracquart P, Guimont C, Linden G. A 16 kDa protein family overexpressed by *Streptococcus thermophilus* PB18 in acid environments. Microbiol. 1997;143:1587–94.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis. 2000;21:1037–53.

Gouesbert G, Jan G, Boyaval P. *Lactobacillus delbrueckii* subsp. *bulgaricus* thermotolerance. Lait. 2001;81:301–9.

Gouesbet G, Jan G, Boyaval P. Two-dimensional electrophoresis study of *Lactobacillus delbrueckii* subsp *bulgaricus* thermotolerance. APP Environmental Microbio. 2002;68:1055–63.

Graves PR, Haystead TAJ. Molecular biologist's guide to proteomics. Microbiol Molecul Biol Rev. 2002;66:39–63.

Guerrera IC, Kleiner O. Application of mass spectrometry in proteomics. Biosci Rep. 2005;25:71–93.

Guillaume E, Berger B, Affolter M, Kussmann M. Label-free quantitative proteomics of two *Bifidobacterium longum* strains. J Proteomics. 2009;72:771–84.

Guillot A, Gitton C, Anglade P, Mistou MY. Proteomic analysis of *Lactococcus lactis*, a lactic acid bacterium. Proteomics. 2003;3:337–54.

Gupta MK, Subramanian V, Yadav JS. Immunoproteomic identification of secretory and subcellular protein antigens and functional evaluation of the secretome fraction of *Mycobacterium immunogenum*, a newly recognized species of the Mycobacterium chelonae-Mycobacterium abscessus group. J Proteome Res. 2009;8:2319–30.

Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc Natl Acad Sci USA. 2000;97:9390–5.

Hamon E, Horvatovich P, Izquierdo E, Bringel F, Marchioni E, Aoudé-Werner D, Ennahar S. Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance. BMC Microbiol. 2011;11:63.

Hansen MC, Nielsen AK, Molin S, Hammer K, Kilstrup M, Palmer RJ Jr, Udsen C, White DC. Changes in rRNA levels during stress invalidates results from mRNA blotting: fluorescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels. J Bacteriol. 2001;183:4747–51.

Harder A, Wildgruber R, Nawrocki A, Fey SJ, Larsen PM, Görg A. Comparison of yeast cell protein solubilization procedures for two-dimensional. Electrophoresis. 1999;20:826–9.

Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn L, Bindels JG, Welling GW. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr. 2000;30:61–7.

Hartke A, Bouché S, Giard JC, Benachour A, Boutibonnes P, Auffray Y. The lactic lcid stress response of *Lactococcus lactis* subsp *lactis*. Curr Microbiol. 1996;33:194–9.

Hartke A, Frere J, Boutibonnes P, Auffray Y. Differential induction of the chaperonin GroEL and the Co-chaperonin GroES by heat, acid, and UV-irradiation in *Lactococcus lactis* subsp. *lactis*. Curr Microbiol. 1997;34:23–6.

Hartl DL, Jones EW. Genetics: analysis of Genes and genomes. Boston: Jones and Bartlett Publishers; 2005.

Haynes PA, Yates JR. Proteome profiling-pitfalls and progess. Yeast. 2000;17:81–7.

Herve-Jimenez L, Guillouard I, Guedon E, Gautier C, Boudebbouze S, Hols P, Monnet V, Rul F, Maguin E. Physiology of *Streptococcus thermophilus* during the late stage of milk fermentation with special regard to sulfur amino-acid metabolism. Proteomics. 2008;8:4273–86.

Herve-Jimenez L, Guillouard I, Guedon E, Boudebbouze S, Hols P, Monnet V, Maguin E, Rul F. Postgenomic analysis of *Streptococcus thermophilus* cocultivated in milk with *Lactobacillus delbrueckii* subsp. *bulgaricus*: involvement of nitrogen, purine, and iron metabolism. Appl Environ Microbiol. 2009;75:2062–73.

Hofmann AF. Bile acids: the good, the bad, and the ugly. News Physiol Sci. 1999;14:24–9.

Hörmann S, Scheyhing C, Behr J, Pavlovic M, Ehrmann M, Vogel RF. Comparative proteome approach to characterize the high-pressure stress response of *Lactobacillus sanfranciscensis* DSM 20451(T). Proteomics. 2006;6:1878–85.

Hurdle JG, O'Neill AJ, Chopra I, Lee RE. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol. 2011;9:62–75.

Irmler S, Raboud S, Beisert B, Rauhut D, Berthoud H. Cloning and characterization of two *Lactobacillus casei* genes encoding a cystathionine lyase. Appl Environ Microbiol. 2008;74:99–106.

Isolauri E, Salminen S, Ouwehand AC. Probiotics. Best Pract Res Clin Gastroenterol. 2004;18:299–313.

Izquierdo E, Horvatovich P, Marchioni E, Aoude-Werner D, Sanz Y, Ennahar S. 2-DE and MS analysis of key proteins in the adhesion of *Lactobacillus plantarum*, a first step toward early selection of probiotics based on bacterial biomarkers. Electrophoresis. 2009;30:949–56.

Jacobsen CN, Nielsen VR, Hayford AE, Moller PL, Michaelsen KF, Paerregaard A, Sandstrom B, Tvede M, Jakobsen M. Screening probiotic activities of 47 strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Appl Environ Microbiol. 1999;65:4949–56.

James P, Quadroni M, Carafoli E, Gonnet G. Protein identification by mass profile fingerprinting. Biochem Biophys Res Commun. 1993;195:58–64.

Jensen ON, Podtelejnikov AV, Mann M. Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. Anal Chem. 1997;69:4741–50.

Joo WA, Kim CW. Proteomics of *Halophilic archaea*. J Chromatography B. 2005;2:237–50.

Jordan KN, Cogan TM. Heat resistance of *Lactobacillus* spp. isolated from cheddar cheese. Lett Appl Microbiol. 1999;29:136–40.

Kanehisa M, Goto S. Kyoto encyclopedia of genes and genomes: KEGG. Nucleic Acids Res. 2000;28:27–30.

Kelly P, Maguire PB, Bennett M, Fitzgerald DJ, Edwards RJ, Thiede B, Treumann A, Collins JK, O'Sullivan GC, Shanahan F, Dunne C. Correlation of probiotic *Lactobacillus salivarius* growth phase with its cell wall-associated proteome. FEMS Microbiol Lett. 2005;252:153–9.

Keshava Prasad TS, Goel R, Kandasamy K, Keerthikumar S, Kumar S, Mathivanan S, Telikicherla D, Raju R, Shafreen B, Venugopal A, Balakrishnan L, Marimuthu A, Banerjee S, Somanathan DS, Sebastian A, Rani S, Ray S, Harrys Kishore CJ, Kanth S, Ahmed M, Kashyap MK, Mohmood R, Ramachandra YL, Krishna V, Rahiman BA, Mohan S, Ranganathan P, Ramabadran S, Chaerkady R, Pandey A. Human protein reference Database-2009 update. Nucleic Acids Res. 2009;37:D767–72.

Kilstrup M, Jacobsen S, Hammer K, Vogensen FK. Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*. Appl Environ Microbiol. 1997;63:1826–37.

Kim SW, Dunn NW. Identification of a cold shock gene in lactic acid bacteria and the effect of cold shock on cryotolerance. Curr Microbiol. 1997;35:59–63.

Kim SW, Ren J, Dunn NW. Differentiation of *Lactococcus lactis* subspecies *lactis* and subspecies *cremoris* strains by their adaptive response to stresses. FEMS Microbiol. 1999;171:57–65.

Koch S, Eugster-Meier E, Oberson G, Meile L, Lacroix C. Effects of strains and growth conditions on autolytic activity and survival to freezing and lyophilization of *Lactobacillus delbrueckii* ssp. *lactis* isolated from cheese. Int Dairy J. 2008;18:187–96.

Koponen J, Laakso K, Koskenniemi K, Kankainen M, Savijoki K, Nyman TA, de Vos WM, Tynkkynen S, Kalkkinen N, Varmanen P. Effect of acid stress on protein expression and phosphorylation in *Lactobacillus rhamnosus* GG. J Proteomics. 2012;75:1357–74.

Koskenniemi K, Laakso K, Koponen J, Kankainen M, Greco D, Auvinen P, Savijoki K, Nyman TA, Surakka A, Salusjärvi T, de Vos WM, Tynkkynen S, Kalkkinen N, Varmanen P. Proteomics and transcriptomics characterization of bile stress response in probiotic *Lactobacillus rhamnosus* GG. Mol Cell Proteomics. 2011;10(M110):002741.

Krishna RG, Wold F. Post-translational modification of proteins. Adv Enzymol Relat Areas Mol Biol. 1993;67:265–98.

Kuipers R, Leer R, Tarchini SA, Peters HM, Sandbrink MW, Fiers EJ, Stiekema E, Lankhorst RM, Bron PA, Hoffer RM, Groot MN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ. Complete genome sequence of *Lactobacillus plantarum*. Proc Natl Acad Sci USA. 2003;100:1990–5.

Kuwana R, Yamamoto N. Increases in GroES and GroEL from *Lactobacillus acidophilus* L-92 in response to a decrease in medium pH, and changes in cytokine release from splenocytes: transcriptome and proteome analyses. J Biosci Bioeng. 2012;114:9–16.

Lamberti C, Pessione E, Giuffrida MG, Mazzoli R, Barello C, Conti A, Giunta C. Combined cup loading, bis(2-hydroxyethyl) disulfide, and protein precipitation protocols to improve the alkaline proteome of *Lactobacillus hilgardii*. Electrophoresis. 2007;28:1633–8.

Laplace JM, Sauvageot N, Harke A, Auffray Y. Characterization of *Lactobacillus collinoides* response to heat, acid and ethanol treatments. Appl Microbiol Biotechnol. 1999;51:659–63.

Larsen NM, Boye H, Siegumfeldt M, Jakobsen M. Differential expression of proteins and genes in the lag phase of *Lactococcus lactis* subsp. *lactis* grown in synthetic medium and reconstituted skim milk. Appl Environ Microbiol. 2006;72:1173–9.

Lau AT, He QY, Chiu JF. Proteomic technology and its biomedical application. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai). 2003;35:965–75.

Lavermicocca P, Valerio F, Lonigro SL, Angelis MD, Morelli L, Callegari ML, Rizzello CG, Visconti A. Study of adhesion and survival of lactobacilli and bifidobacteria on table olives with the aim of formulating a new probiotic food. Appl Environ Microbiol. 2005;71:4233–40.

Le Marrec C, Bon E, Lonvaud-Funel A, McLeod A. Tolerance to high osmolality of the lactic acid bacterium *Oenococcus oeni* and identification of potential osmoprotectants. Int J Food Microbiol. 2007;115:335–42.

Lebeer S, Vanderleyden J, De Keersmaecker SCJ. Genes and molecules of Lactobacilli supporting probiotic action. Microbiol Mol Biol Rev. 2008;72:728–64.

Lee J, Kaletunc G. Evaluation of the heat inactivation of *Escherichia coli* and *Lactobacillus plantarum* by differential scanning calorimetry. Appl Environ Microbiol. 2002;68:5379–86.

Lee K, Pi K. Effect of transient acid stress on the proteome of intestinal probiotic *Lactobacillus reuteri*. Biochemistry. 2010;75:460–5.

Lee K, Lee HG, Pi K, Choi YG. The effect of low pH on protein expression by the probiotic bacterium *Lactobacillus reuteri*. Proteomics. 2008;8:1624–30.

Lee JY, Pajarillo EA, Kim MJ, Chae JP, Kang DK. Proteomic and transcriptional analysis of *Lactobacillus johnsonii* PF01 during bile salt exposure by iTRAQ shotgun proteomics and quantitative RT-PCR. J Proteome Res. 2013;12:432–43.

Len ACL, Harty DWS, Jacques NA. Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance. Microbiol. 2004;150:1339–51.

Leverrier P, Dimova D, Pichereau V, Auffray Y, Boyaval P, Jan G. Leverrier, Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: physiological and proteomic analysis. Appl Environ Microbiol. 2003;69:3809–18.

Leverrier P, Vissers JP, Rouault A, Boyaval P, Jan G. Mass spectrometry proteomic analysis of stress adaptation reveals both common and distinct response pathways in *Propionibacterium freudenreichii*. Arch Microbiol. 2004;181:215–30.

Lim EM, Ehrlich DS, Maguin E. Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electrophoresis. 2000;21:2557–61.

Link AJ, Eng J, Schieltz DM, Carmack E. Direct analysis of protein complexes using mass spectrometry. Nat Biotechnol. 1999;17:676–82.

Ljungh A, Wadstrom T. *Lactobacillus* molecular biology from genomics to probiotics. Caister Academic Press. *Lactobacillus* stress responses. UK: Norfolk; 2009. pp. 115–138.

Lorca G, de Valdez F. A Low-pH-Inducible, stationary-phase acid tolerance response in *Lactobacillus acidophilus* CRL 639 G.L. Curr Microbiol. 2001;42:21–5.

Lundeen SG, Savage DC. Multiple forms of bile salt hydrolase from *Lactobacillus* sp. strain 100–100. J Bacteriol. 1992;174:7217–20.

Maddalo G, Chovanec P, Stenberg-Bruzell F, Nielsen HV, Jensen-Seaman MI, Kline KA, Daley DO. A reference map of the membrane proteome of *Enterococcus Faecalis*. Proteomics. 2011;11:3935–41.

Majumder A, Sultan A, Jersie-Christensen RR, Ejby M, Schmidt BG, Lahtinen SJ, Jacobsen S, Svensson B. Proteome reference map of *Lactobacillus acidophilus* NCFM and quantitative proteomics towards understanding the prebiotic action of lactitol. Proteomics. 2011;11:3470–81.

Majumder A, Cai L, Ejby M, Schmidt BG, Lahtinen SJ, Jacobsen S, Svensson B. Two-dimensional gel-based alkaline proteome of the probiotic bacterium *Lactobacillus acidophilus* NCFM. Proteomics. 2012;12:1006–14.

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Daz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G,

Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D. Comparative genomics of the lactic acid bacteria. J Bacteriol. 2007;189:1199–208.

Mann M, Hojrup P, Roepstorff P. Use of mass spectrometric molecular weight information to identify proteins in sequence databases. Biol Mass Spectrom. 1993;22:338–45.

Marceau A, Zagorec M, Chaillou S, Méra T, Champomier-Vergès M. Evidence for involvement of at least six proteins in adaptation of *Lactobacillus sakei* to cold temperatures and addition of NaCl. Appl Environ Microbiol. 2004a;12:7260–8.

Margolles A, Garcia L, Sánchez B, de los Reyes-Gavilan CG. Characterisation of a Bifidobacterium strain with acquired resistance to cholate-a preliminary study. Int J Food Microbiol. 2003;82:191–8.

Marles-Wright J, Lewis RJ. Stress responses of bacteria. Curr Opin Struct Biol. 2007;17:755–60.

Martín R, Sánchez B, Suárez JE, Urdaci MC. Characterization of the adherence properties of human Lactobacilli strains to be used as vaginal probiotics. FEMS Microbiol Lett. 2012;328:166–73.

McLeod A, Zagorec M, Champomier-Verges M, Naterstad K, Axelsson L. Primary metabolism in *Lactobacillus sakei* food isolates by proteomic analysis. BMC Microbiol. 2010;10:120.

Minellia EB, Beninia A, Marzottob M, Sbarbatic A, Ruzzenented O, Ferrarioe R, Hendriksf H, Dellagliob F. Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional dairy foods. Int Dairy J. 2004;14:723–36.

Molenaar D, Hagting A, Alkema H, Driessen AJM, Konings WN. Characteristics and osmoregulatory roles of uptake systems for proline and glycine-betaine in *Lactococcus lactis*. J Bacteriol. 1993;175:5438–44.

Monedero V, Maze′ A, BoeL G, Zu′ñiga M, Beaufils S, Hartke A, Deutscher J. The phosphotransferase system of Lactobacillus casei: regulation of carbon metabolism and connection to cold shock response. J Mol Microbiol Biotechnol. 2007;12:20–32.

Neville BA, O'Toole PW. Probiotic properties of *Lactobacillus salivarius* and closely related *Lactobacillus* species. Future Microbiol. 2010;5:759–74.

Nitisinprasert S, Pungsungworn N, Wanchaitanawong P, Loiseau G, Montet D. In vitro adhesion assay of lactic acid bacteria, *Escherichia coli* and. *Salmonella* sp. by microbiological and PCR methods. J Sci Technol. 2006;28:99–106.

O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem. 1975;250:4007–21.

Obis D, Guillot A, Gripon JC, Renault P, Bolotin A, Mistou MY. Genetic and biochemical characterization of a high affinity betaine uptake system (BusA) in *Lactococcus lactis* reveals a new functional organization within bacterial ABC transporters. J Bacteriol. 1999;181:6238–46.

Obis D, Guillot A, Mistou MY. Tolerance to high osmolality of *Lactococcus lactis* subsp. *lactis* and *cremoris* is related to the activity of a betaine transport system. FEMS Microbiol Lett. 2001;202:39–44.

Oh HK, Lee JY, Lim SJ, Kim MJ, Kim GB, Kim JH, Hong SK, Kang DK. Molecular cloning and characterization of a bile salt hydrolase from *Lactobacillus acidophilus* PF01. J Microbiol Biotechnol. 2008;18:449–56.

O'Sullivan E, Condon S. Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. Appl Environ Microbiol. 1997;63:4210–5.

Ouwehand AC, Tuomola EM, Tolkko S, Salminen S. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. Int J Food Microbiol. 2001;64:119–26.

Panoff JM, Thammavongs B, Laplace JM, Hartke A, Boutibonnes P, Boutibonnes Y, Auffray Y. Cryotolerance and Cold adaptation in *Lactococcus lactis* subsp. *lactis* IL1403. Cryobiology. 1995;32:516–20.

Pappin DD, Hojrup JP, Bleasby AJ. Rapid identification of proteins by peptide-mass finger printing. Curr Biol. 1993;3:327–32.

Paul CD, Colin H. Surviving the acid test: responses of gram-positive bacteria to low pH. Curr Microbiol Mol Biol Rev. 2003;67:429–53.

Payne CM, Crowley C, Washo-Stultz D, Briehl M, Bernstein H, Bernstein C, Beard S, Holubec H, Warneke J. The stress-response proteins poly(ADP-ribose) polymerase and NF-κB protect against bile salt-induced apoptosis. Cell Death Diff. 1998;5:623–36.

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. 1999;20:3551–67.

Perrin C, Guimont C, Bracquart P, Gaillard JL. Expression of a new cold shock protein of 21.5 kDa and of the major cold shock protein by *Streptococcus thermophilus* after cold shock. Curr Microbiol. 1999;39:342–7.

Perrin C, Gonzalez-Marquez H, Gaillard JL, Guimont C. Reference map of soluble proteins from *Streptococcus thermophilus* by two-dimensional electrophoresis. Electrophoresis. 2000;21:949–55.

Pessione E, Mazzoli R, Giuffrida MG, Lamberti C, Garcia-Moruno E, Barello C, Conti A, Giunta C. A proteomic approach to studying biogenic amine producing lactic acid bacteria. Proteomics. 2005;5:687–98.

Pessione A, Lamberti C, Pessione E. Proteomics as a tool for studying energy metabolism in lactic acid bacteria. Mol Biosyst. 2010;6:1419–30.

Pessione A, Lamberti C, Cocolin L, Campolongo S, Grunau A, Giubergia S, Eberl L, Riedel K, Pessione E. Different protein expression profiles in cheese and clinical isolates of *Enterococcus faecalis* revealed by proteomic analysis. Proteomics. 2012;12:431–47.

Phadtare S, Yamanaka K, Inouye M. The cold shock response. In: Stortz G, Hengge-Aronis R, editors. Bacterial stress response. Washington, DC: ASM Press; 2000. p. 33–46.

Pichereau V, Bourot S, Flahaut S, Blanco C, Auffray Y, Bernard T. The osmoprotectant glycine betaine inhibits salt-induced cross-tolerance towards lethal treatment in *Enterococcus faecalis*. Microbiol. 1999;145:427–35.

Piuri M, Sánchez-Rivas C, Ruzal SM. Adaptation to high salt in *Lactobacillus*: role of peptides and proteolytic enzymes. J Appl Microbiol. 2003;95:372–9.

Piuri M, Sánchez-Rivas C, Ruzal SM. Cell wall modifications during osmotic stress in *Lactobacillus casei*. J Appl Microbiol. 2005;98:84–95.

Plumed-Ferrer C, Koistinen KM, Tolonen TL, Lehesranta SJ, Kärenlampi SO, Mäkimattila E, Joutsjoki V, Virtanen V, von Wright A. Comparative study of sugar fermentation and protein expression patterns of two *Lactobacillus plantarum* strains grown in three different media. Appl Environ Microbiol. 2008;74:5349–58.

Prasad J, McJarrow P, Gopal P. Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. Appl Environ Microbiol. 2003;69:917–25.

Quivey RG Jr, Faustoferri RC, Clancy KA, Marquis RE. Acid adaptation in *Streptococcus mutans* UA159 alleviates sensitization to environmental stress due to RecA deficiency. FEMS Microbiol Lett. 1995;126:257–61.

Rabilloud T. A comparison between low background silver diammine and silver nitrate protein stains. Electrophoresis. 1992;13:429–39.

Randazzo CL, Restuccia C, Romano AD, Caggia C. *Lactobacillus casei*, dominant species in naturally fermented sicilian green olives. Int J Food Microbiol. 2004;90:9–14.

Rechinger KB, Siegumfeldt H, Svendsen I, Jakobsen M. "Early" protein synthesis of *Lactobacillus delbrueckii* ssp. *bulgaricus* in milk revealed by [^{35}S] methionine labeling and two-dimensional gel electrophoresis. Electrophoresis. 2000;21:2660–9.

Reddy KBPK, Awasthi SP, Madhu AN, Prapulla SG. Role of cryoprotectants on the viability and functional properties of probiotic lactic acid bacteria during freeze drying. Food Biotechnol. 2009;23:243–65.

Reuter G. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. Intest Microbiol. 2001;2:43–53.

Roos S, Karner F, Axelsson L, Jonsson H. *Lactobacillus mucosae* sp. nov., a new species with in vitro mucus-binding activity isolated from pig intestine. Int J System Evol Microbiol. 2000;50:251–8.

Ruiz L, Couté Y, Sánchez B, de los Reyes-Gavilán CG, Sánchez JC, Margolles A. The cell-envelope proteome of Bifidobacterium longum in an in vitro bile environment. Microbiol. 2009a;155:957–67.

Ruiz L, Sánchez B, de Los Reyes-Gavilán CG, Gueimonde M, Margolles A. Coculture of Bifidobacterium longum and Bifidobacterium breve alters their protein expression profiles and enzymatic activities. Int J Food Microbiol. 2009b;133:148–53.

Rycroft CE, Jones MR, Gibson GR, Rastall RA. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. J Appl Microbiol. 2001;91:878–87.

Salminen S, Isolauri E. Intestinal colonization, microbiota, and probiotics. J Pediatr. 2006;149:S115–20.

Salminen S, Laine M, von Wright A, Vuopio-Varkila J, Korhonen T, Mattila-Sandholm T. Development of selection criteria for probiotic strains to assess their potential in function foods: a Nordic and European approach. Biosci Microflora. 1996;15:61–7.

Sánchez B, Champomier-Vergès MC, Anglade P, Baraige F, de Los Reyes-Gavilán CG, Margolles A, Zagorec M. Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. J Bacteriol. 2005;187:5799–808.

Sánchez B, Champomier-Vergès MC, Collado Mdel C, Anglade P, Baraige F, Sanz Y, de los Reyes-Gavilán CG, Margolles A, Zagorec M. Low-pH Adaptation and the acid tolerance response of *Bifidobacterium longum*. Appl Environ Microbiol. 2007a;73:6450–9.

Sánchez B, Champomier-Vergès MC, Stuer-Lauridsen B, Ruas-Madiedo P, Anglade P, Baraige F, de los Reyes-Gavilán CG, Johansen E, Zagorec M, Margolles A. Adaptation and response of Bifidobacterium animalis subsp. lactis to bile: a proteomic and physiological approach. Appl Environ Microbiol. 2007b;73:6757–67.

Santoni V, Molloy M, Rabilloud T. Membrane proteins and proteomics: un amour impossible? Electrophoresis. 2000;21:1054–70.

Savijoki K, Suokko A, Palva A, Valmu L, Kalkkinen N, Varmanen P. Effect of heat-shock and bile salts on protein synthesis of *Bifidobacterium longum* revealed by [^{35}S] methionine labeling and two-dimensional gel electrophoresis. FEMS Microbiol Lett. 2005;248:207–15.

Savijoki K, Suokko A, Palva A, Varmanen P. New convenient defined media for [^{35}S] methionine labelling and proteomic analyses of probiotic lactobacilli. Lett Appl Microbiol. 2006;42:202–9.

Savijoki K, Lietzén N, Kankainen M, Alatossava T, Koskenniemi K, Varmanen P, Nyman TA. Comparative proteome cataloging of *Lactobacillus rhamnosus* strains GG and Lc705. J Proteome Res. 2011;10:3460–73.

Scheifer KH, Ludwig W. Phylogeny of the genus *Lactobacillus* and related genera. System Appl Microbiol. 1995;18:461–7.

Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore PD, Arigoni F. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci USA. 2002;99:14422–7.

Schmidt G, Hertel C, Hammes WP. Molecular characterization of the *danK* operon of *Lactobacillus sakei* LTH681. Syst Appl Microbiol. 1999;22:321–8.

Shaw MM, Riederer BM. Sample preparation for two-dimensional gel electrophoresis. Proteomics. 2003;3:1408–17.

Sheehan VM, Sleator RD, Fitzgerald GF, Hill C. Heterologous expression of BetL, a betaine uptake system, enhances the stress tolerance of *Lactobacillus salivarius* UCC118. Appl Environ Microbiol. 2006;3:2170–7.

Sheehan VM, Sleator RD, Hill C, Fitzgerald GF. Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain *Bifidobacterium breve* UCC2003. Microbiology. 2007;153:3563–71.

Shin HY, Lee JH, Lee JY, Han YO, Han MJ, Kim DH. Purification and characterization of ginsenoside Ra-hydrolyzing β-D-xylosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium. Biol Pharm Bull. 2003;26:1170–3.

Sieuwerts S, Molenaar D, van Hijum SA, Beerthuyzen M, Stevens MJ, Janssen PW, Ingham CJ, de Bok FA, de Vos WM, van Hylckama Vlieg JE. Mixed-culture transcriptome analysis reveals the molecular basis of mixed-culture growth in Streptococcus thermophilus and Lactobacillus bulgaricus. Appl Environ Microbiol. 2010;76:7775–84.

Singh OV, Nagaraj NS. Transcriptomics, proteomics and interactomics: unique approaches to track the insights of bioremediation. Brief Funct Genomic Proteomic. 2006;4:355–62.

Smelt JP, Otten GD, Bos AP. Modelling the effect of sublethal injury on the distribution of the lag times of individual cells of *Lactobacillus plantarum*. Int J Food Microbiol. 2002;73:207–12.

Sneath PHA, Mair NS, Sharpe ME. Bergey's manual of systematic bacteriology, vol. 2. Baltimore: Williams & Wilkins; 1986. p. 1209–45.

Somero GN. Proteins and temperature. Annu Rev Physiol. 1995;57:43–68.

Sperandio B, Polard P, Ehrlich DS, Renault P, Guedon E. Sulfur amino acid metabolism and its control in *Lactococcus lactis* IL1403. J Bacteriol. 2005;187:3762–78.

Stiles EM, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. Int J Food Microbiol. 1997;36:1–29.

Streit F, Delettre J, Corrieu G, Beal C. Acid adaptation of *Lactobacillus delbreckii* subsp. *bulgaricus* induces physiological responses at membrane and cytosolic levels that improves cryotolerance. J Appld Microbiol. 2008;105:1071–80.

Strupat K, Karas M, Hillenkamp F, Eckerskorn C, Lottspeich F. Matrix-assisted laser desorption ionization mass spectrometry of proteins electroblotted after polyacrylamide gel electrophoresis. Anal Chem. 1994;66:464–70.

Sun Z, Bo X, He X, Jiang Z, Wang F, Zhao H, Liu D, Yuan J. Comparative proteome analysis of *Bifidobacterium longum* NCC2705 grown on fructose and glucose. Sheng Wu Gong Cheng Xue Bao. 2008;24:1401–6.

Svensater G, Sjogreen B, Hamilton IR. Multiple stress responses in *Streptococcus mutans* and the induction of general and stress specific proteins. Microbiol. 2000;146:107–17.

Teixera P, Castro H, Mohacsi-Farkas C, Kirby R. Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. J Appl Microbiol. 1997;83:219–26.

Thammavongs B, Corroler D, Panoff JM, Auffray Y, Boutibonnes P. Physiological response of *Enterococcus faecalis* JH2-2 to cold shock: growth at low temperatures and freezing/thawing challenge. Lett Appl Microbiol. 1996;23:398–402.

Tsakalidou E, Papadimitriou K. Stress responses of lactic acid bacteria. LLC: Springer New York Dordrecht Heidelberg London. Springer Science & Business Media; 2011. p. 67–90.

Twyman R. Proteomics. http://www.genome.wellcome.ac.uk/doc_wtd020767.html. Accessed 2003.

Tymczyszyn EE, Gómez-Zavaglia A, Disalvo EA. Influence of the growth at high osmolality on the lipid composition, water permeability and osmotic response of *Lactobacillus bulgaricus*. Arch Biochem Biophys. 2005;443:66–73.

Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis. 1997;18:2071–7.

Van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek. 2002;82:187–216.

Van Der Heide T, Poolman B. Glycine betaine transport in *Lactococcus lactis* is osmotically regulated at the level of expression and translocation activity. J Bacteriol. 2000;182:203–6.

Van Velkinburgh JC, Gunn JS. PhoP-PhoQ regulated loci are required for enhanced bile resistance in *Salmonella* spp. Infect Immun. 1999;67:1614–22.

Van Wijk KJ. Challenges and prospects of plant proteomics. Plant Physiol. 2001;126:501–8.

Vitali B, Wasinger V, Brigidi P, Guilhaus M. A proteomic view of *Bifidobacterium infantis* generated by multi-dimensional chromatography coupled with tandem mass spectrometry. Proteomics. 2005;5:1859–67.

Wang Y, Delettre J, Guillot A, Corrieu G, Beal C. Influence of cooling temperature and duration ocold adaptation of *Lactobacillus acidophilus* RD758. Cryobiol. 2005;50:294–307.

Wang J, Guo Z, Zhang Q, Yan L, Chen W, Liu XM, Zhang H. Fermentation characteristics and transit tolerance of probiotic *Lactobacillus casei* Zhang in soymilk and bovine milk during storage. J Dairy Sci. 2008;92:2468–76.

Wang J, Zhang W, Zhong Z, Wei A, Bao Q, Zhang Y, Sun T, Postnikoffa A, Meng H, Zhang H. Gene expression profile of probiotic *Lactobacillus casei* Zhang during the latestage of milk fermentation. Food Control. 2011a;25:321–7.

Wang, J, Zhang W, Zhong Z, Wei A, Bao Q, ZhangY, Sun T, Postnikoffa A, Meng H, Zhang H. Transcriptome analysis of probiotic Lactobacillus casei Zhang during fermentation in soymilk. J Ind Microbial Biotechnol. 2011b;39:191–206.

Wang J, Wu R, Zhang W, Sun Z, Zhao W, Zhang H. Proteomic comparison of a new probiotic bacterium *Lactobacillus casei* Zhang cultivated in milk and soymilk. J Dairy Sci. 2013;96:5603–24.

Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I. Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. Electrophoresis. 1995;16:1090–4.

Westermeier R, Naven T, Höpker HR. Proteomics strategies. In: Proteomics in practice: a guide to successful experimental design. 2nd ed. KGaA, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co; 2008.

Whitehead K, Versalovic J, Roos S, Britton RA. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. Appl Environ Microbiol. 2008;74:1812–9.

Wilkins MR, Sánchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, Williams KL. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev. 1996;13:19–50.

Wilkins JC, Homer KA, Beighton D. Altered protein expression of *Streptococcus oralis* cultured at low pH revealed by two-dimensional gel electrophoresis. Appl Environ Microbiol. 2001;67:3396–405.

Wilkins JC, Homer KA, Beighton D. Analysis of *Streptococcus mutans* proteins modulated by culture under acidic conditions. Appl Environ Microbiol. 2002;68:2382–90.

Wouters JA, Sanders JW, Kok J, de Vos WM, Kuipers OP, Abee T. Clustered organization and transcriptional analysis of a family of five *csp* genes of *Lactococcus lactis* MG1363. Microbiology. 1998;144:2885–93.

Wouters JA, Rombouts FM, de Vos WM, Kuipers OP, Abee T. Cold shock proteins and low-temperature response of *Streptococcus thermophilus* CNRZ302. Appl Environ Microbiol. 1999a;65:4436–42.

Wouters JA, Jeynov B, Rombouts FM, de Vos WM, Kuipers OP, Abee T. Analysis of the role of 7 kDa cold-shock proteins of *Lactococcus lactis* MG1363 in cryoprotection. Microbiology. 1999b;145:3185–94.

Wouters JA, Rombouts FM, Kuipers OP, de Vos WM, Abee T. The role of cold-shock proteins in low-temperature adaptation of food-related bacteria. Syst Appl Microbiol. 2000a;23:165–73.

Wouters JA, Mailhes M, Rombouts FM, de Vos WM, Kuipers OP, Abee T. Physiological and regulatory effects of controlled overproduction of five cold shock proteins of *Lactococcus lactis* MG1363. Appl Environ Microbiol. 2000b;66:3756–63.

Wu R, Wang W, Yu D, Zhang W, Li Y, Sun Z, Wu J, Meng H, Zhang H. Proteomics analysis of *Lactobacillus casei* Zhang, a new probiotic bacterium isolated from traditional home-made koumiss in inner Mongolia of China. Mol Cell Proteomics. 2009a;8:2321–38.

Wu R, Wang L, Wang J, Li H, Menghe B, Wu J, Guo M, Zhang H. Isolation and preliminary probiotic selection of lactobacilli from koumiss in inner Mongolia. J Basic Microbiol. 2009b;49:318–26.

Wu R, Sun Z, Wu J, Meng H, Zhang H. Effect of bile salts stress on protein synthesis of *Lactobacillus casei* Zhang revealed by 2-dimensional gel electrophoresis. J Dairy Sci. 2010;93:3858–68.

Wu R, Zhang W, Sun T, Wu J, Yue X, Meng H, Zhang H. Proteomic analysis of responses of a new probiotic bacterium *Lactobacillus casei* Zhang to low acid stress. Int J Food Microbiol. 2011;147:181–7.

Wu C, Zhang J, Chen W, Wang M, Du G, Chen J. A combined physiological and proteomic approach to reveal lactic-acid-induced alterations in *Lactobacillus casei* Zhang and its mutant with enhanced lactic acid tolerance. Appl Microbiol Biotechnol. 2012;93:707–22.

Wu J, Zhang J, Shi P, Wu R, Yue X, Zhang H. Bacterial community involved in traditional fermented soybean paste dajiang made in northeast China. Ann Microbio. 2013;63:1417–21.

Xie Y, Chou LS, Cutler A, Weimer B. DNA macroarray profiling of *Lactococcus lactis* subsp. *lactis* IL 1403 gene expression during environmental stresses. Appl Environ Microbiol. 2004;70:6738–47.

Yang F, Wang J, Li X, Ying T, Qiao S, Li D, Wu G. 2-DE and MS analysis of interactions between *Lactobacillus fermentum* I5007 and intestinal epithelial cells. Electrophoresis. 2007;28:4330–9.

Yates JR. Mass spectrometry and the age of the proteome. J Mass Spectrom. 1998;33:1–19.

Yates JR 3rd, Speicher S, Griffin PR, Hunkapiller T. Peptide mass maps: a highly informative approach to protein identification. Anal Biochem. 1993;214:397–408.

Yoon KY, Woodams EE, Hang YD. Production of probiotic cabbage juice by lactic acid bacteria. Bioresour Technol. 2006;97:1427–30.

Yuan J, Zhu L, Liu X, Li T, Zhang Y, Ying T, Wang B, Wang J, Dong H, Feng E, Li Q, Wang J, Wang H, Wei K, Zhang X, Huang C, Huang P, Huang L, Zeng M, Wang H. A proteome reference map and proteomic analysis of *Bifidobacterium longum* NCC2705. Mol Cell Proteomics. 2006;5:1105–18.

Zaidi AH, Bakkes PJ, Lubelski J, Agustiandari H, Kuipers OP, Driessen AJM. The ABC-Type multidrug resistance transporter LmrCD is responsible for an extrusion-based mechanism of bile acid resistance in *Lactococcus lactis*. J Bacteriol. 2008;190:7357–66.

Zapparoli G. Colony dimorphism associated with stress resistance in *Oenococcus oeni* VP01 cells during stationary growth phase. FEMS Microbiol Lett. 2004;239:261–5.

Zavaglia AG, Kociubinsky G, Perez P, de Antoni G. Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. J Food Prot. 1998;61:865–73.

Zhang W, Chait BT. ProFound: an expert system for protein identification using mass spectrometric peptide mapping information. Anal Chem. 2000;72:2482–9.

Zhang W, Yu D, Sun Z, Wu R, Chen X, Chen W, Meng H, Hu S, Zhang H. Complete genome sequence of *Lactobacillus casei* Zhang, a new probiotic strain isolated from traditional homemade koumiss in Inner Mongolia (China). J Bacteriol. 2010a;192:5268–9.

Zhang YH, Zhang YP, Zhu Y, Mao S, Li Y. Proteomic analyses to reveal the protective role of glutathionein resistance of *Lactococcus lactis* to osmotic stress. Appl Environ Microbiol. 2010b;1:3177–86.

Zhao J, Cheung PC. Comparative proteome analysis of *Bifidobacterium longum* subsp. *infantis* grown on β-glucans from different sources and a model for their utilization. J Agic Food Chem. 2013;61:4360–70.

Zink R, Walker C, Schmidt G, Elli M, Pridmore D, Reniero R. Impact of multiple stress factors on the survival of dairy lactobacilli. Sci Aliments. 2000;20:119–26.

Chapter 5
Lactic Acid Bacteria in Health and Disease

Jinzhong Xiao, Yong Zhang and Zhennai Yang

Abstract Probiotics are defined as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host." The consumption of probiotics or probiotics-containing products is able to relieve clinical symptoms in patients with intestinal disorders, inflammatory bowel disease, irritable bowel syndrome, type-2 diabetes, metabolic syndrome, and allergic diseases. Currently, various probiotic products are available in the market. Among these products, probiotic dairy products including pasteurized milk, fermented milks, cheeses, dairy beverages, dried products, ice-cream, and other dairy desserts account for the largest percentage of probiotic foods. Dairy products have been considered as an ideal food vehicle for delivering probiotic microorganisms to the human gastrointestinal tract. Other nondairy products including plant-based products such as cereals, fruits, and vegetables containing no cholesterol with low allergic reactions, and meat products are also being increasingly used for development of probiotic products. Probiotic microorganisms show variations in survival and growth ability in different food substrates due to different processing technologies and storage conditions. Prebiotics or plant components can be added to different types of probiotic products to improve the probiotic viability and physicochemical properties of the products. This chapter mainly provides information on the definition, the criteria of a good probiotic, and the beneficial effects upon probiotic

J. Xiao (✉)
Food Science and Technology Institute, Morinaga Milk Industry Co. Ltd, Tokyo, Japan
e-mail: j_xiao@morinagamilk.co.jp

Z. Yang
Beijing Technology and Business University, Beijing, China
e-mail: yangzhennai@th.btbu.edu.cn

Y. Zhang
Research Center for Nutrition and Food Safety, The Third Military Medical University, Chongqing, People's Republic of China
e-mail: zhy-545@163.com

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_5,
© Springer Science+Business Media Dordrecht 2014

administration, as well as presents an overview on the manufacturing technology of probiotic products, including methods of probiotic addition, effects of processing and storage conditions on probiotic viability, and functional evaluation of probiotic products.

Keywords Lactic acid bacteria · Health · Disease

5.1 Lactic Acid Bacteria as Probiotics

5.1.1 Introduction

It has been a longtime since studies on the possible effects of the oral ingestion of microorganisms on promoting health and preventing diseases have achieved the spotlight. Studies conducted at the beginning of the last century suggesting the possible health benefits of microorganisms are believed to be the basis for the development of the concept of probiotics. In “The Prolongation of Life” by Metchinikoff (1907), the authors suggested that Bulgarian populations that consumed large quantities of fermented milk had longevity. In 1905, Tissier observed that children with diarrhea had a lower number of *Bifidobacteria* in their stool than healthy children, and he suggested that these bacteria could be administered to patients with diarrhea to help restore a healthy gut flora (Tissier 1905). In addition, many studies have been conducted on the health benefits of microorganisms, including intestinal microbiota and those existing in traditionally fermented foods or natural sources. In particular, lactic acid bacteria (LAB), including *Lactobacillus* and *Bifidobacterium,* have been the targets of much of the research conducted. Meanwhile, continuous interest has been given to the study of the association between microbiota and health conditions and disease development. In the past two decades, a number of exciting findings demonstrated that changes in microbiota are related to disorders such as allergy and cancer, gastrointestinal inflammatory disorders (e.g., Crohn’s disease and ulcerative colitis), obesity, diabetes, and brain health. Today, the use of probiotics in maintaining and promoting health is gaining much attention, and the market of probiotic products continues to expand.

5.1.1.1 Definition of Probiotics

The word probiotics is derived from two Greek words meaning “for life.” Lilley and Stillwell (1965) first used the term probiotic to mean “a microbial substance that stimulates the growth of another microorganism.” In 1974, Parker first used the word probiotic in the context of animal feed supplementation and defined it as

"organisms and substances with beneficial effects for animals via a modification of intestinal microbial balance" (Parker 1974). In 1989, Fuller redefined probiotics by focusing on microorganisms: "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1989). This definition stresses the need for the supplement to be composed of viable microorganisms and has been the most common definition of probiotics for many years.

The need for an international consensus on the methodology to assess the efficacy and safety of probiotics triggered joint Food and Agricultural Organization and the World Health Organization (FAO/WHO) projects that succeeded in providing guidelines for the evaluation of probiotics (Food and Agriculture Organization of the United Nations and World Health Organization 2001, 2002) and came to a recommendation for the adoption of the definition of probiotics as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host" (Food and Agriculture Organization of the United Nations and World Health Organization 2002). This definition provided a simple and easily understood description of probiotics; however, there are some issues on the criteria and evaluation methods for probiotics that need further discussion.

5.1.1.2 Criteria of Probiotics

In order to qualify as probiotic, microorganisms should be alive when administered, must be taxonomically defined at a species level, be demonstrated to be safe for its intended use, and be evaluated and documented for health benefits in the target host. From such a point of view, a bacterial isolate or a product containing bacteria should not be considered as probiotics if the species of the microorganisms is unknown and there is not any documented health benefits. As a probiotic bacterium, the organism themselves must be specified using appropriate methods and given a designation, usually called strain number, e.g., *Lactobacillus (L.) rhamnosus* GG. In addition, the bacteria need to be viable in sufficient quantity at time of ingestion to confer the health benefit.

An international standard for probiotics as a component in food is currently not established. Table 5.1 lists LAB intended as food additives as listed by the European Food Safety Authority (EFSA) as qualified presumption of safety (QPS), in Canada (as live microorganisms (probiotics), and in China (as microorganisms for normal food use). Concerning strains of *Enterococcus,* in the opinion of EFSA, this species will no longer be assessed for QPS unless new scientific information becomes available, since there is increasing evidence of pathogenicity (EFSA 2011).

In some countries, there are special regulations for the use of probiotics in infant foods. In China, there is a special list of probiotic strains for use in foods for infants and children less than 3 years old. Currently, the list contains six strains, three of them belong to the *Bifidobacterium* family and the remainder to *Lactobacillus*. In other countries, including the United States, the European Union (EU),

Table 5.1 List of lactic acid bacteria for intentional adding in food

Genus	Species	EFSA[a]	Canada[b]	China[c]
Bifidobacterium	*adolescentis*	+	+	+
	animalis[d]	+	+	+
	bifidum	+	+	+
	breve	+	+	+
	longum[e]	+	+	+
Lactobacillus	*acidophilus*	+	+	+
	alimentarius	+		
	amylolyticus	+		
	amylovorus	+	+	
	aviaries	+		
	brevis	+		
	buchneri	+		
	casei	+	+	+
	cellobiosus	+		
	collinoides	+		
	coryniformis	+		
	crispatus	+		+
	curvatus	+		
	delbrueckii[f]	+	+[g]	+
	farciminis	+		
	fermentum	+	+	+
	gallinarum	+		
	gasseri	+	+	+
	helveticus	+		+
	hilgardii	+		
	johnsonni	+	+	+
	kefiranofaciens	+		
	kefiri	+		
	mucosae	+		
	panis	+		
	paracasei	+	+	+
	paraplantarum	+		
	pentosus	+		
	plantarum	+	+	+
	pontis	+		
	reuteri	+	+	+
	rhamnosus	+	+	+
	sakei	+		
	salivarius	+	+	+
	sanfranciscensis	+		
Lactococcus	*lactis*	+		+
Leuconostoc	*citreum*	+		
	lactis	+		
	mesenteroides	+		+
Oenococcus	*oeni*	+		

(continued)

Table 5.1 (continued)

Genus	Species	EFSA[a]	Canada[b]	China[c]
Pediococcus	*acidilactici*	+		
	dextrinicus	+		
	pentosaceus	+		
Streptococcus	*thermophilus*	+	+	+

[a] The 2011 updated list of QPS recommended biological agents (EFSA 2007)
[b] Live microorganisms (probiotics) list in Canada. (http://webprod.hc-sc.gc.ca/nhpid-bdipsn/atReq.do?atid=live.micro&lang=eng)
[c] List of lactic acid bacteria for food use in China. (http://sps.saic.gov.cn/zcfg/201102/t20110228_104291.html)
[d] Including *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*
[e] Including *B. longum* subsp. *infantis*
[f] Including *L. bulgaricus* subsp. *lactis*
[g] Only for subsp. *bulgaricus*

Canada, and Japan, strains for infant use are not distinguished from those used in normal foods. However, in the United States, GRAS (generally recognized as safe) approval by the Food and Drug Administration (FDA) for a specific strain is a prerequisite for intended use in infant formula. In Japan, current government regulations do not allow for the addition of any probiotics to infant formula.

5.1.2 Screening and Evaluation of Probiotics

Probiotics must be able to exert their benefits on the host (Collins et al. 1998; Morelli 2000), and activity is suggested to be strain-specific. Figure 5.1 shows a general procedure for isolation and characterization of novel strains with presumed probiotic features.

5.1.2.1 Sources for Bacterial Strains

It is suggested that an effective human probiotic should be a normal intestinal inhabitant for humans. For this reason, most of the probiotic strains for human use originate from human intestines. Recent studies have shown that there are more than 400 species of organisms, with a total of more than 10^{14} bacteria in the human gastrointestinal tract. For the isolation of novel strains, classical cultivation techniques must be employed. Since the human gastrointestinal tract is generally a neutral and anaerobic environment, isolates of commensal bacteria tend to be relatively more sensitive to low pH and oxygen compared to those from sources of plant materials or the animal gastrointestinal tract. These characteristics may encompass some technical hardness for industrial application, such as keeping a high survivability in fermented milk.

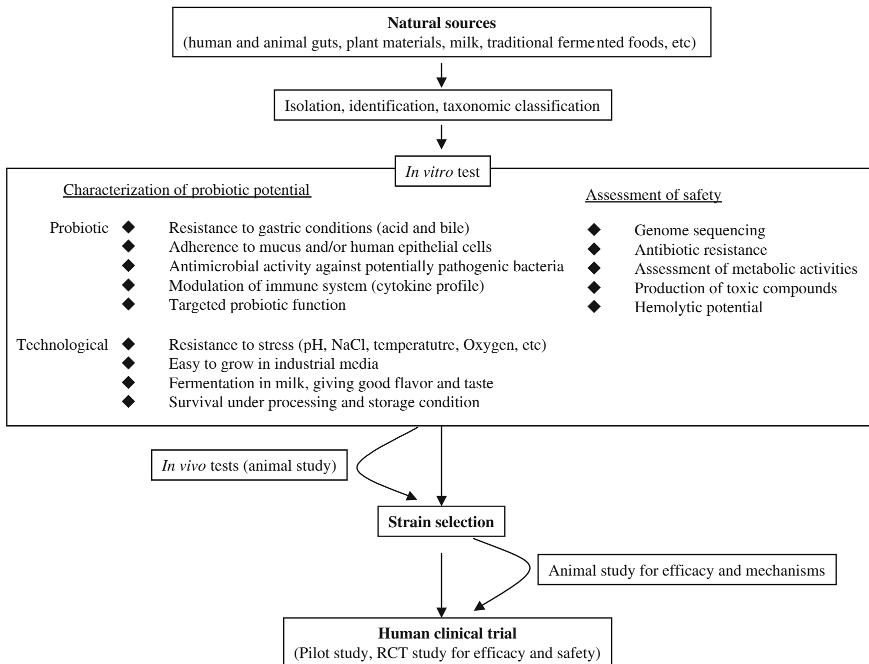


Fig. 5.1 Procedures for the isolation and characterization of novel strains with presumed probiotic features

However, there were arguments that it is the specificity of the action, not the source of the microorganism that is important. There are other sources of screening of probiotic strains, for example, animal intestines, plant material, raw milk, human breast milk, and traditionally fermented foods (Collins et al. 1998; Wu et al. 2009b).

The progress of genomic and molecular technology enable improvements or 'design' probiotic characteristics toward specific therapies (Hazebrouck et al. 2007). However, the use of recombinant strains is still far from being applied in functional foods, at least in the EU and Japan, although there is the potential for medical application.

5.1.2.2 Identification of Strains

Probiotics must be taxonomically identified at both the genus and species level, which is important to link a strain to a specific health effect as well as safety. In particular, it is currently believed that probiotic effects are strain-specific.

Identification of the bacterium can be performed based on the most current valid methodology. Currently, comparison of the DNA sequences encoding 16S and 23S rRNA has been demonstrated to be the most powerful and labor-saving genetic tool for identification of many bacteria species (Tilsala-Timisjärvi and Alatossava 1997), however, it is suggested to use a combination of phenotypic and genetic tests in some cases. Recently, various genotype-based methods have been adopted as useful ways for the identification of bacteria (Amann et al. 1995). Single polymerase chain reaction (PCR) methods, using primers that target variable regions in universal genes, such as the 16S rRNA, 16S-23S rRNA intergenic spacer region (ISR), or 23S rRNA, have successfully detected and identified the LAB (Kim et al. 2005; Nour 1998; Ward and Timmins 1999). Moreover, the multiplex PCR methodologies, using primers that target different genes, have been shown to be a useful tool to quickly identify multiple bacteria in a single reaction (Kwon et al. 2005; Settanni et al. 2005; Odamaki et al. 2011a, b; Sul et al. 2007). On the other hand, patterns generated from the fermentation of a range of sugars and final fermentation products obtained from glucose utilization are key phenotypes that should be investigated for identification purposes.

5.1.2.3 Functional Evaluation

Evidence relating to the effects of probiotics on human health can be obtained from in vitro, in vivo or clinical studies. In vitro or in vivo studies in animals can be useful in advancing our understanding of the mechanisms whereby probiotics exert their effects. Table 5.2 shows a list of the main currently used in vitro tests for the study of probiotic strains recommended by the Working Group (FAO/WHO 2002). However, it must be understood that these tests are not fully adequate to predict the potential benefits of probiotic microorganisms in the human body, and there are only a few probiotic strains in the market, which has been characterized, by all of these features. Among them, in vitro test for resistance to bile salts has been shown to correlate with in vivo gastric survival and may be useful (Conway et al. 1987). There are some animal models that may provide substantiation of in vitro effects and determination of probiotic mechanism. However, because of the vastly increased complexity in living systems and inter-individual variation, clinical studies in human are needed to validate probiotic efficacy.

5.1.2.4 Safety Evaluation

Safety is an important issue of probiotics. Probiotics may theoretically be responsible for some side effects, such as causing systemic infections, generating deleterious metabolic activities, triggering excessive immune stimulation in susceptible individuals, and transferring harmful genes.

Bifidobacterium and *Lactobacillus,* a group of bacteria that is generally considered as safe, are among the primary candidates of probiotics (Salminen et al.

Table 5.2 In vitro tests for the study of probiotic strains

In vitro tests
Resistance to gastric acidity and bile acid
Ability to adherence to mucus and/or human epithelial cells and cell lines
Antimicrobial or ability to reduce pathogen adhesion activity against potentially pathogenic bacteria
Bile salt hydrolase activity
Resistance to spermicides (applicable to probiotics for vaginal use)

1998). Currently, no cases of infection have been reported with *Bifidobacterium*. There are two reports on infection caused by *L. rhamnosus* from the use of commercial products (Mackay et al. 1999; Rautio et al. 1999). On the other hand, *Enterococcus* is suggested to be risk of causing nosocomial infections. In the guidelines for evaluating probiotics recommended by FAO/WHO (2001), the necessary for determination of antibiotic resistance characteristics and assessment of certain metabolic activities, such as D-lactate production, bile salt deconjugation were suggested. In addition, it was suggested to be important to carefully assess the side effects during human studies and make epidemiological observation of any incidence of adverse effects in consumers (post-marketing). If the strain under evaluation belongs to a species that is a known mammalian toxin producer or with known hemolytic potential, the possibility for producing toxin or having hemolytic activity must be tested following appropriate procedure (SCAN 2000). Moreover, assessment of lack of infectivity using immunocompromised animal model is suggested be a measure of adding confidence to the safety of the probiotic strain.

The resistance of bacteria to antibiotics is an increasingly important public health problem worldwide. Bacteria which contain transmissible drug resistance genes, such as genes located in chromosome with relation to transposon or plasmid were suggested to be not suitable for use in foods. The QPS approach for systemic screening of antibiotic resistance has been launched by the EFSA (Fig. 5.2, European Food Safety Authority 2008, http://www.efsa.europa.eu/en/efsajournal/doc/732.pdf), which is useful to determine the resistance to the most relevant antibiotics for each strain used as probiotics in food or feed additives. Table 5.3 shows the Microbial Break Points (MBPs) of bacteria related to probiotic property defined by EFSA Panel on additives and products or substances used in animal feed (EFSA 2012), which is useful for the evaluation of the antibiotic susceptibility of probiotic strains. Moreover, the availability of bacterial genome sequences and many databases constructed based on the available genomic information have brought sequence-based identification approaches for safety evaluation, such as antibiotic resistance and potential of toxin production in probiotics (Wei et al. 2012).

In some cases, studies using animal models will be useful to provide evidence for both health benefits and safety evaluation as well as to determine the mechanisms. There are safety concerns related to the risk of bacterial translocation and the development of septicemia, especially for infants and immunocompromised individuals. Instances of probiotic-induced sepsis have been reported in infants

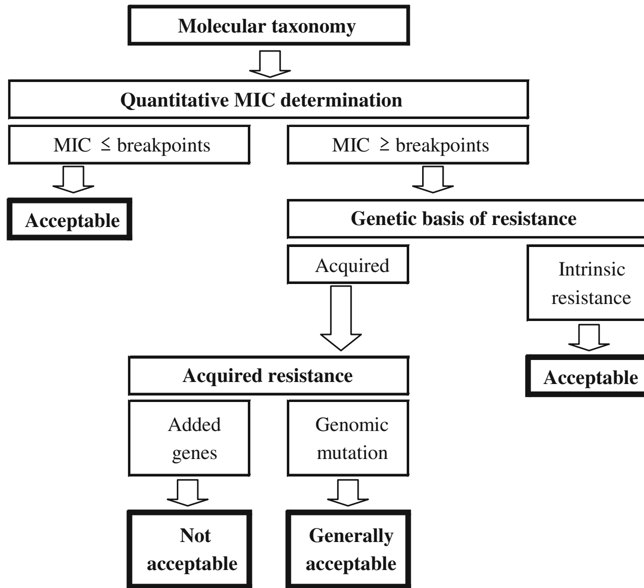


Fig. 5.2 Proposed schemes for antibiotic resistance assessment of a bacterial strain (European Food Safety Authority 2008, http://www.efsa.europa.eu/en/efsajournal/doc/732.pdf)

(Land et al. 2005; Ohishi et al. 2010). Therefore, it is important to assess the safety in clinical trials; however, it needs attention that clinical trials may not have sufficient numbers of subjects to detect rare but adverse effects.

5.1.3 Health Benefits of Lactobacilli *and* Bifidobacteria

Historically, LAB were believed to have health benefits such as improving nutrient digestion, alleviating symptoms of lactose intolerance and producing functional substances in the gastrointestinal tract. Lactose malabsorption is a result of insufficient activity of lactase in the human gut which leads to abdominal distension, excessive flatulence, and/or diarrhea. The β-galactosidase of LAB is suggested to contribute to tolerance to lactose.

Commensal bacteria are known as a significant source of some vitamins (Hill 1997). *Bifidobacteria* are generally thought to synthesize several B family vitamins, including folate, biotin, thiamine, nicotinic acid, pyridoxine, riboflavin, and cyanocobalamin. The ability to produce vitamins is known to be species-dependent for *Bifidobactium* (Pompei et al. 2007). The vitamins produced by colonic bacteria are suggested to contribute to systemic vitamin levels and especially to the homeostasis of vitamins in the localized epithelial cells of large intestine (Said and Mohammed 2006). Animal and clinical studies found that supplementation with

Table 5.3 Microbiological cut-off values (mg/L)

	Ampicillin	Vancomycin	Gentamicin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol	Tylosine
Lactobacillus obligate homofermentative[a]	1	2	16	16	16	1	1	4	4	n.r.
Lactobacillus obligate heterofermentative[b]	2	n.r.	16	32	64	1	1	8	4	n.r.
Lactobacillus facultative heterofermentative[c]	4	n.r.	16	64	64	1	1	8	4	n.r.
Lactobacillus acidophilus group	1	2	16	64	16	1	1	4	4	n.r.
Lactobacillus reuteri	2	n.r.	8	64	64	1	1	16	4	n.r.
Lactobacillus plantarum/pentosus	2	n.r.	16	64	n.r.	1	2	32	8	n.r.
Lactobacillus rhamnosus	4	n.r.	16	64	32	1	1	8	4	n.r.
Lactobacillus casei/paracasei	4	n.r.	32	64	64	1	1	4	4	n.r.
Bifidobacterium	2	2	64	n.r.	128	1	1	8	4	n.r.
Pediococcus	4	n.r.	16	64	64	1	1	8	4	n.r.
Leuconostoc	2	n.r.	16	16	64	1	1	8	4	n.r.
Lactococcus lactis	2	4	32	64	32	1	1	4	8	n.r.
Streptococcus thermophilus	2	4	32	64	64	2	2	4	4	n.r.
Bacillus species	n.r.	4	4	8	8	4	4	8	8	n.r.
Propionibacterium	2	4	64	64	64	0.5	0.25	2	2	n.r.
Enterococcus faecium	2	4	32	1,024	128	4	4	4	16	4
Other *Gram* positive bacteria	1	2	4	16	8	0.5	0.25	2	2	n.r.

n.r. not required
[a] including *L. delbrueckii* and *L. helveticus*
[b] including *L. fermentum*
[c] including the homofermentative species *L. salivarius*

Bifidobacteria of potential folate-producing led to increased plasma folate level in rats and in increased folate concentrations in feces in humans, confirming the in vivo vitamin production and absorption by the hosts (Rossi et al. 2011).

In addition, probiotics have been proven to improve intestinal health such as reducing or preventing diarrhea, improving constipation, balancing intestinal microbiota, stimulating the immune system, preventing or alleviating allergic disorders, improving lipid metabolism and reducing the risk or suppressing the development of cancers. As a result of the accumulative evidence of health benefits, probiotics are currently used worldwide as foods or dietary supplements for maintaining health condition as well as alternative medicines in the prevention and treatment of diseases, specifically of the intestinal disorders. However, these effects may be strain-dependent.

5.1.3.1 Intestinal Disorders

Acute diarrhea caused by infection is a significant health problem worldwide which causes several million of death each year. In developing countries, the majority of deaths are children. Food-borne diarrhea response to the major problem of diarrhea in developed countries. There are many studies for the effect of probiotics in the treatment or prevention of acute diarrhea. A systematic review of published RCT trials for preventing or treating acute diarrhea in infants and children showed that probiotics significantly reduced risk of diarrhea (Szajewska and Mrukowicz 2001). A subsequent meta-analysis of 18 eligible studies indicated that bacterial probiotic therapy shortens the duration of acute diarrheal illness in children by approximately 1 day (Huang et al. 2002).

Antibiotic treatment is frequently accompanied by the appearance of diarrhea, i.e., antibiotic-associated diarrhea (AAD) that often caused by *Clostridium difficile,* particularly in population of hospitalized elderly peoples (≥65 years) exposed to broad-spectrum antibiotics. When caused by *C. difficile*, AAD can result in life-threatening illness. *Clostridium difficile* is not uncommon in a healthy gastrointestinal tract, but antibiotics treatments cause the disruption of the indigenous microbiota which leads to an abnormal proliferation of the bacteria and subsequent increased production of toxin. A systematic review and meta-analysis on 20 randomized, controlled trials including adult and pediatric patients with 3818 participants showed that probiotic treatment reduced the incidence of *C. difficile*-associated diarrhea (CDAD) by 66 %, suggesting that probiotic prophylaxis results in a large reduction in CDAD without an increase in clinically important adverse events (Johnston et al. 2012). However, Allen et al. (2013) recently reported that they identified no evidence of a multistrain preparation of *Lactobacilli* and *Bifidobacteria* as a preventative for AAD or CDAD in older hospitalized patients in a large scale (2981 patients), randomized, double-blind, placebo-controlled, multicenter trial. These results suggest the need for further evaluation of the clinical effects as well as an improved understanding of the mechanisms.

Hospitalized elderly, particular for those receiving enteral feedings, are known to have significant problems with defecation with possible consequences of constipation or diarrhea. Randomized, placebo-controlled trials in elderly patients receiving enteral feeding showed that ingestion of *Bifidobacterium (B.) longum* BB536 improved bowel movements for patients with a low as well as a high frequency of defecation, leading to a normal defecation frequency (Kondo et al. 2013b). This suggests the potential of *B. longum* BB536 in improving the health care of elderly patients receiving enteral feeding.

Constipation featured as difficulty in passing stool, with excessive hardness of stool or slow transit through the bowel is a major problem for young women and elderly. Many studies have been performed to test the effect of probiotic treatment, however, the outcome is controversial and the effect is suggested to be a feature of selected strains. Consumption of milk or yoghurt supplemented with *B. longum* BB536 resulted in an increased frequency of defecation and increased cell numbers and relative percentages of *Bifidobacteria* and decreased concentrations of ammonia and beta-glucuronidase in fecal samples (Ogata et al. 1997; Yaeshima et al. 1997). Further randomized, placebo-controlled studies are needed to explore the effects of probiotics in the treatment of constipation.

The human gut is colonized by trillions of bacteria composed of hundreds of species. The cell numbers of gut microbiota is larger than that of human body and accumulative findings strongly support a profound influence on human physiology, immunology, and nutrition from the residential microbes (Bäckhed et al. 2005; Sekirov et al. 2010; Kau et al. 2011). For instance, an imbalance in the gut microbiota is linked to various disorders such as obesity, inflammatory bowel disease, metabolic syndrome, diabetes, and colon cancer. Therefore, controlling the balance of the intestinal microbiota may be one of the most effective means to maintain good health in humans. There are many studies on the effects of prevention of infection via regulating the balance of intestinal microbiota by probiotic administration. Enterohemorrhagic *Escherichia coli* (EHEC), which is also known as Shiga toxin-producing *E. coli*, is the most common enteropathogen worldwide that colonizes the large intestine and induces hemorrhagic colitis, diarrhea, hemolytic uremic syndrome, and encephalopathy. Recently, *Lactobacillus casei* Zhang was confirmed to keep the innate immune system alert by increasing the transcription of toll-like receptors (TLRs) in macrophages which may contribute to against potential infections (Wang et al. 2012b). There are several reports on probiotic inhibition of pathogenic *E. coli* O157 by *Bifidobacterium* or *Lactobacillus* in gnotobiotic mice (Ogawa et al. 2001; Namba et al. 2003; Asahara et al. 2004; Momose et al. 2005; Yoshimura et al. 2010). By using a gnotobiotic mouse model, Fukuda et al. (2011) applied a multi-omics approach to elucidate the molecular basis of the probiotic effect of different species and strains of *Bifidobacterium* on O157 lethal infection and found that this effect can be attributed, at least in part, to an increased production of acetate. This increased level of acetic acid led to the inhibition of translocation of the Shiga toxin from the lumen to the blood. These studies demonstrated the potential of probiotics in the prevention of EHFC infection, however, due to practical issues, it is difficult to elucidate effect in

human studies. Recently, Ouwehand et al. (2013) made a challenge to test the clinical effect of probiotics on infection with ETEC, by giving a live, but attenuated, ETEC vaccine to healthy men, which induced mild, short-lived symptoms. However, they found that supplementation with *Lactobacillus acidophilus* ATCC 700396 was ineffective in reducing ETEC infection symptoms.

On the other hand, there are some enterotoxigenic bacteria in our gut microbiota. Some strains of *Bacteroides fragilis*, namely "enterotoxigenic *B. fragilis*" (ETBF), were known to secrete a 20-kDa zinc-dependent metalloprotease toxin (BFT). Enterotoxigenic *B. fragilis* asymptomatically colonize 10–40 % of the human population, and is also known as a cause of diarrheal diseases in animal and human. A possible association of ETBF with colorectal cancer has been demonstrated from clinical observations and animal models using multiple intestinal neoplasia mice (Toprak et al. 2006; Wu et al. 2009a). In a pilot study, Odamaki et al. showed that oral ingestion of a yoghurt containing probiotic *B. longum* BB536 for 8 weeks significantly decreased the gut cell number of ETBF (Odamaki et al. 2012).

These results imply the potential of probiotics in regulating gut microbiota, although the mechanism needs to be evaluated. The effect of probiotics is believed to be not only an influence on the composition of microbiota, interesting is being paid to know the changes of microbiota-associated metabolism for both the microbiota and the hosts.

5.1.3.2 Inflammatory Bowel Disease and Irritable Bowel Syndrome

Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, have been suspected for relation with immunological etiology for many years. The effects of probiotics for treating IBD diseases have been studied in animal models and patients with IBD. Evidence from animal models of IBD indicates that probiotics can alter intestinal microbiota and ameliorate disease (Sartor 2004). The clinical efficacy of probiotics in IBD has been investigated in a number of clinical studies and has been comprehensively reviewed (Hedin et al. 2007). Studies have performed using single strain or combination of multistrains including *Bifidobacteria*, lactobacilli, and others (e.g., *E. coli* Nissle 1917). Supplementation of fermented milk containing *Bifidobacteria* has been shown to be effective in the management of active ulcerative colitis (Kato et al. 2004). A randomized, placebo-controlled study including 36 patients showed the efficacy of the administration of VSL#3 (a mixture containing eight species of lactobacilli, *Bifidobacteria* and streptococcus), in maintaining antibiotic-introduced remission for at least 1 year in patients with recurrent or refractory pouchitis (Mimura et al. 2004). Supplementation of a probiotic strain, *B. longum* BB536, for 24 weeks alleviated the symptoms of patients with ulcerative colitis (Takeda et al. 2009).

In contrast to the generally achieved clinical efficacy in treatment of ulcerative colitis by probiotics, the effect in the treatment Crohn's disease was not always

promised (De Greef et al. 2013). Reduction in disease activity has been achieved in pediatric patients with Crohn's disease by probiotic intervention (Gupta et al. 2000). However, supplementation of *L. rhamnosus* GG was unable to prevent the recurrence or ameliorate disease severity following colon resection in Crohn's patients (Prantera et al. 2002; Schultz et al. 2004).

Protecting/suppressing intestinal inflammation is suggested to be one of the mechanisms of effects in the treatment of IBD by probiotics. The effects can be categorized as (1) improvement of epithelial barrier function (2) immunomodulating activities, and (3) suppression of the growth or epithelial binding/invasion of pathogenic bacteria (Sartor 2004).

Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is another intestinal disorder featured with frequent symptoms as abdominal pain, bloating, flatulence, and diarrhea. IBS is the result of an abnormal mucosal function owing to an altered immune state accompanied by a persistent, mild inflammatory reaction in the intestine.

Immune system in IBS patients may be modulated by probiotics. It was reported that VSL#3 significantly reduced abdominal bloating compared to the use of a placebo (Kim et al. 2003). A meta-analysis of randomized controlled trials suggested that some probiotics may be effective in the treatment of IBS diseases (Ortiz-Lucas et al. 2013). O'Mahony et al. (2005) showed that patients with IBS have an abnormal baseline interleukin (IL)-10 to IL-12 ratio (the ratio of anti- to pro-inflammatory cytokines), and administration of *Bifidobacterium infantis* 35624 alleviated IBS symptoms. The alleviating effects of *B. infantis* 35624 were suggested to be associated with the normalization of this ratio of IL-10 to IL-12. It is thought that the mechanisms supporting these beneficial effects are the combination of such immune effects, changes in the intraluminal milieu, and secondary effects on colonic transit (Camilleri 2006). However, as IBS is a heterogeneous disorder, further clinical studies are needed.

5.1.3.3 Effects on Immune Function and Anti-infective Actions

The human immune system can be separated as nonspecific immunity (innate) and specific immunity (adaptive), both of which function to eradicate foreign microorganisms such as viruses and bacteria. Probiotic usage has been shown to affect both innate immunity such as eliciting phagocytes and natural killer (NK) cells (Takeda and Okumura 2007; Gill et al. 2001) as well as adaptive immunity such as promoting the secretion of immunoglobulin (Ig) A into feces and saliva (Kotani et al. 2010; Kabeerdoss et al. 2011) and enhancing the effects of influenza vaccination (Olivares et al. 2007).

The common cold is an upper respiratory tract infection and is one of the most common diseases occurring in all ages. Influenza is an acute viral respiratory

disease caused by influenza virus, which attacks the host respiratory tract mucosa. Some studies have revealed that probiotics have a preventive effect against common cold and influenza virus infection. Probiotic administration has been shown to reduce the incidence and duration of infection in children and adults during the winter season (De Vrese et al. 2005; Leyer et al. 2009).

The decline of immune function increases risk of morbidity and mortality from common infections in elderly. It is known that elderly people are poor responder to vaccination. In a preliminary study, Namba et al. (2010) reported that continuous ingestion of *B. longum* BB536 reduced the incidence of influenza infection and fever in the elderly. In a double-blind, placebo-controlled study in elderly patients receiving enteral tube feedings, ingestion of probiotic *B. longum* BB536 for 12 weeks significantly increased the population of *Bifidobacteria* in the intestinal microbiota and potentiated both innate and adaptive immune function (Akatsu et al. 2013). NK cell activity decreased significantly in the placebo group during the intervention, but not in the *B. longum* BB536 group (Akatsu et al. 2013).

There is evidence that intestinal microbes greatly influence IgA production in the mucosa (Macpherson et al. 2008). Secretary IgA antibodies are the major effectors molecules in the mucosal immune system and play a crucial role in the first defense against infection. Oral administration of *Bifidobacteria* has been reported to induce the production of mucosal total IgA and antigen-specific IgA in mice (Takahashi et al. 1998). Tejada-Simon et al. (1999) showed that administration of yoghurt supplemented with *L. acidophilus* and *Bifidobacterium* spp. enhanced mucosal and systemic IgA responses to the cholera toxin immunogen. A tendency for increases in serum IgA level was observed in the *B. longum* BB536 group compared to the placebo group (Akatsu et al. 2013).

Recently, a meta-analysis on 10 randomized, controlled trials with 2894 participants (1588 in the probiotics group and 1306 in the control group) concluded that probiotics may have a modest effect on the prevention of the common cold (Kang et al. 2013).

5.1.3.4 Effects on Allergic Diseases

The prevalence of allergic diseases such as eczema, allergic rhinitis, and asthma has rapidly increased worldwide over the past several decades, especially in industrialized countries. One explanation is the "hygiene hypothesis," which suggests that decreased exposure to immunostimulating agents such as pathogenic bacteria in early childhood leads to a unbalanced maturation of the immune system which result in an increased sensitivity to allergenic substances (Strachan 1989). Bacterial components have been shown to affect the differentiation and development of T cells via TLRs (Kaisho and Akira 2006). In general, one of the most accepted theory is that probiotic can regulate the balance between Th1 and Th2 cytokines production. Ya et al. (2008) reported that *L. casei* Zhang could influence the development of Th1 and Th2 homeostasis via suppressing Th1-related TNF-α and IL-2 secretion in mice. There are numerous studies demonstrating the benefits

of probiotics in the preventing and/or treating allergic diseases, however, the efficacy is still controversial.

Current evidence suggests that the benefits of probiotics are more promised in the prevention of allergic diseases. As a pioneering study, the Finish group demonstrated the effect by prenatal administration of *L. rhamnosus* GG to mothers and postnatal administration to their infants in the primary prevention of development of eczema in children (Kalliomäki et al. 2001). Similar studies were followed by several groups (Kukkonen et al. 2007; Abrahamsson et al. 2007; Ou et al. 2012). A meta-analysis based on 14 studies demonstrated that probiotic use decreased the incidence of atopic dermatitis and provided evidence in support of a moderate role of probiotics in the prevention of atopic dermatitis and IgE-associated atopic dermatitis in infants (Pelucchi et al. 2012). Similarly, a meta-analysis by Elazab et al. (2013) suggested that prenatal and/or early life probiotic administration reduces the risk of atopic sensitization and decreases total IgE levels in children but may not reduce the risk of asthma or wheezing. They found that administration of *L. acidophilus* was associated with an increased risk of atopic sensitization ($P = 0.002$) compared with other strains, which suggests the importance of probiotic strain selection (Elazab et al. 2013). However, with regard to the efficacy of the clinical use of probiotics in pediatric allergy, a report performed on behalf of the World Allergy Organization (WAO) Special Committee on Food Allergy and Nutrition concluded that probiotics do not have an established role in the prevention or treatment of allergy (Fiocchi et al. 2012). They suggested that epidemiologic, immunologic, microbiologic, genetic, and clinical studies are necessary to determine whether probiotic supplements will be useful in preventing allergy and that there is a need for collaborations between allergo-immunologists and microbiologists in basic research and a multidisciplinary approach in future clinical research (Fiocchi et al. 2012).

Most of the studies seeking to prevent eczema/atopic diseases in infants have been performed by administering the bacteria to pregnant women and then providing it to their infants. Studies have also been performed to assess the possible benefit by administrating to mothers or infants alone. It was showed that *L. rhamnosus* GG did not affect the risk of eczema in their infants by administrating to pregnant women for 4 weeks before delivery (Boyle et al. 2011). In contrast, studies showed that probiotic supplementation to pregnant women and during breastfeeding resulted in decreased incidence of atopic eczema in infants (Dotterud et al. 2010; Rautava et al. 2012). These studies suggest that maternal administration of probiotics, especially during breastfeeding, may be an effective approach for preventing eczema development in the infants.

On the other hand, West et al. showed that administration of *Lactobacillus paracasei* F19 to infants from 4 to 13 months of age, but not to their mothers, decreased the incidence of eczema (West et al. 2009). In contrast, *L. acidophilus* LAVRI-A1 was demonstrated to negatively influencing incidence of eczema in infants when the probiotics was administrated to infants during the first 6 months of (Taylor et al. 2007; Prescott et al. 2008). Further studies are thus needed to evaluate the benefit of probiotics in the prevention of allergic diseases by

administrating to infants alone, including the timing of probiotic intervention and the strain selection. The effects of probiotics in the prevention of other allergic diseases, such as asthma and rhinitis have only been limitedly reported. One year follow-up evaluation of infants with atopic eczema who have been administrated with a mixture of *Bifidobacterium breve* M-16 V and prebiotics found that the incidence of asthma-like symptoms in infants was significantly reduced in the treated group as compared to the placebo group (Van der Aa et al. 2011). Administration of *L. rhamnosus* HN001 to mothers and their infants was effective in decreasing the incidence of rhinoconjunctivitis in children at ages 4 and 6 years (Wickens et al. 2012, 2013).

Studies have been performed to investigate the long-term effects following early probiotic intervention. It was found that administration of *L. paracasei* F19 during weaning, which resulted in a reduced cumulative incidence of infant eczema, had no effect on any diagnosed allergic disease, airway inflammation or Ig E sensitization at ages 8–9 years (West et al. 2013). Similarly, it was found that the effect of *Lactobacillus reuteri* ATCC 55730 on sensitization and IgE-associated eczema in infancy did not lead to a lower prevalence of respiratory allergic disease at school age (Abrahamsson et al. 2013). These findings suggest that further studies are needed to investigate the long-term benefits of primary prevention of eczema onset by probiotics.

On the other hand, many clinical trials have been conducted to evaluate the therapeutic effects of allergic diseases by probiotics. Meta-analysis suggested that probiotics may have a beneficial, but limited, treatmenting effect by reducing the severity of symptoms of atopic dermatitis and allergic rhinitis (Boyle et al. 2009; Michail et al. 2008; Vliagoftis et al. 2008). The early studies by the Finish group demonstrated that administration of *L. rhamnosus* GG reduced the severity of eczema and atopic eczema in infants with atopic eczema or cow's milk allergy (Majamaa and Isolauri 1997; Isolauri et al. 2000). Ingestion of live or heat-killed *L. paracasei* 33 improved the quality of life for patients with rhinoconjunctivitis (Wang et al. 2004; Peng and Hsu 2005). The efficacy of *B. longum* BB536 in the treatment of Japanese cedar pollinosis (JCPsis) has been confirmed in several clinical trials (Xiao et al. 2006, 2007). Ingestion of *B. longum* BB536 during pollen seasons significantly suppressed the subjective symptom scores compared with the placebo group (Xiao et al. 2006) (Fig. 5.3).

There are only a few studies reporting the clinical effects of probiotics in the treatment of asthma and food allergies. Administration of *Lactobacillus gasseri* A5 improved the symptoms of asthma and rhinitis with changes in immunological parameters (Chen et al. 2010). However, no beneficial effect on asthma-related events was found by administration of *L. casei* DN-114 001 or *L. rhamnosus* GG (Giovannini et al. 2007; Rose et al. 2010). Administration with an extensively hydrolyzed formula supplemented with a combination of *L. casei* CRL431 and *Bifidobacterium lactis* Bb-12 did not improve cow's milk tolerance in infants with cow's milk allergy (Hol et al. 2008). It is necessary to conduct further randomized, placebo-controlled studies to determine whether intervention with probiotics is useful in the treatment of asthma and food allergies.

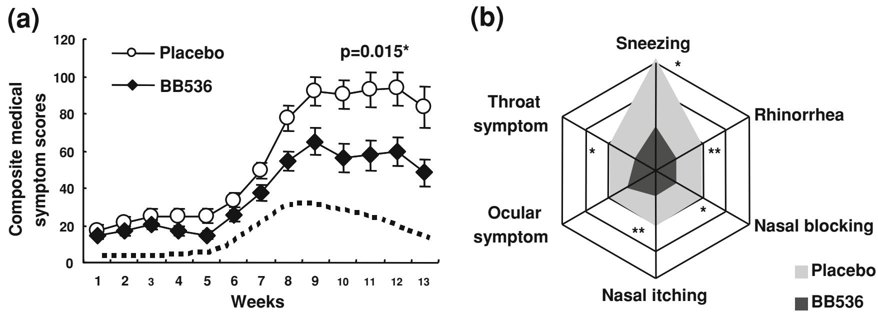


Fig. 5.3 Effect of *Bifidobacterium longum* BB536 intake on the symptoms of Japanese cedar pollinosis. **a** Composite medical symptom scores. P value shown is based on 2-way repeated analysis of variance (ANOVA) on symptom scores between 5–13 weeks, BB536 versus placebo. **b** Area-under-the curves (AUC) of medical scores of each symptom. *P<0.05, **P<0.01, Mann-Whitney U test, BB536 versus placebo

Some reasons have been suggested for the inconsistent conclusions between studies, including the dose and timing of administration, differences in the strain of probiotics used, and underlying host factors such as age, diet, genetics, and microbiota of the host. Consequently, it is crucial to select administration methods and bacterial strains of probiotics, depending upon the type of allergic disease and genetic background of the patient. Although it is quite likely that the efficacy of probiotics depends on the strain, dose, and timing of administration as well as underlying host factors, probiotics can still be a useful approach for the management of allergic diseases.

5.1.3.5 Effects on Metabolic Diseases

Hyperlipidemia and atherosclerosis are the leading causes of the cardiovascular disease and mortality in the world. It was demonstrated that there exists an association between serum cholesterol levels and the risk for coronary heart disease. After Mann (1974) first reported a hypocholesterolemic effect in the Maasai tribesmen that was due to fermented milk of the wild-type starters, several studies have been performed in animals and humans, especially with selected strains of LAB. Recently, a meta-analysis on data from 13 trials, which included 485 participants, showed the potential of probiotic supplementation to lower total and low density lipoprotein (LDL) cholesterol concentrations in plasma among participants with high, borderline-high and normal cholesterol levels (Guo et al. 2011). An update by Aggarwal et al. (2013) also suggested the potential of probiotics in mediating metabolic diseases via the positive modulation of several different physiological systems. In a randomized, double-blind, placebo-controlled, crossover 10-week study, Anderson and Gilliland (1999) showed that daily consumption of 200 g of yoghurt containing *L. acidophilus* L1 after each dinner contributed

to a significant reduction (2.4 %) in serum cholesterol concentration compared to the placebo group. Xiao et al. (2003) evaluated the effects of a low-fat yoghurt containing *B. longum* BL1 on lipid profiles of 32 subjects with moderate hypercholesterolemia. Results from this randomized, single-blind, placebo-controlled, parallel study showed a significant decline in serum total cholesterol, LDL-cholesterol, and triglycerides after 4 weeks.

Several mechanisms of serum lipid improvement have been suggested from in vitro and in vivo studies (Fig. 5.4):

(1) Removal of cholesterol from the intestine. Several studies reported that intestinal LAB have the capacity to absorb cholesterol (Bottazzi et al. 1986; Hosono and Tono-oka 1995). Serum total cholesterol was lowered by declined absorption from the intestine, which led to a reduced concentration in the physiological cholesterol pool.
(2) Deconjugation of bile salt. Some probiotics have been reported to excrete bile salt hydrolase (BSH), the enzyme that catalyzes the hydrolysis of glycine- and taurine-conjugated bile salts into amino acids and bile acids (Grundy 1972). The bile acids are less hydrophilic than the conjugated counterparts, resulting in lower absorption in the intestinal lumen and thus increased excretion into the feces. For maintaining the homeostate of bile acids, cholesterol is used to synthesize new bile acids which result in the lowering of serum cholesterol.
(3) Inhibition of cholesterol synthesis in the liver. Short chain fatty acids (SCFAs) such as acetate and lactate produced by *Lactobacillus* and *Bifidobacterium*, which are reabsorbed from the intestinal lumen and may be transported to liver. Some SCFAs have inhibitory activity against HMG-CoA reductase, a key enzyme for the de novo synthesis of cholesterol (Hara et al. 1998). Microarray analysis of liver revealed that *L. casei* Zhang could accelerate the fatty acid catabolism by enhancing a series of genes involved in β-oxidation of fatty acids and reducing the level of free fatty acid in the liver of hypercholesterolemic rats (Zhong et al. 2012).

Obesity often contributes to the development of cardiovascular diseases, type 2 diabetes, hypertension, certain cancers, and sleep apnea/sleep-disordered breathing. Recent studies have highlighted the role of gut microbiota in the development of obesity and metabolic syndrome (Cani and Delzenne 2009). Bäckhed et al. (2004) found that germ-free mice had about 40 % less total body fat than mice with a normal gut microbiota. Furthermore, conventionalizing germ-free mice with a normal gut microbiota harvested from the cecum of a normal mouse resulted in a 60 % increase in body fat content and insulin resistance within 2 weeks (Bäckhed et al. 2004). Ley et al. (2005) demonstrated that ob/ob mice had a 50 % reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes. The gut microbiota has thus been proposed to participate in regulating energy metabolism by several mechanisms, for example, energy harvesting from the diet, regulating fat storage, regulating lipogenesis, and regulating fatty acid oxidation (Turnbaugh et al. 2006). Recently, Everard et al. (2013) demonstrated that treatment with *Akkermansia muciniphila*, which is a mucin-degrading bacterium that

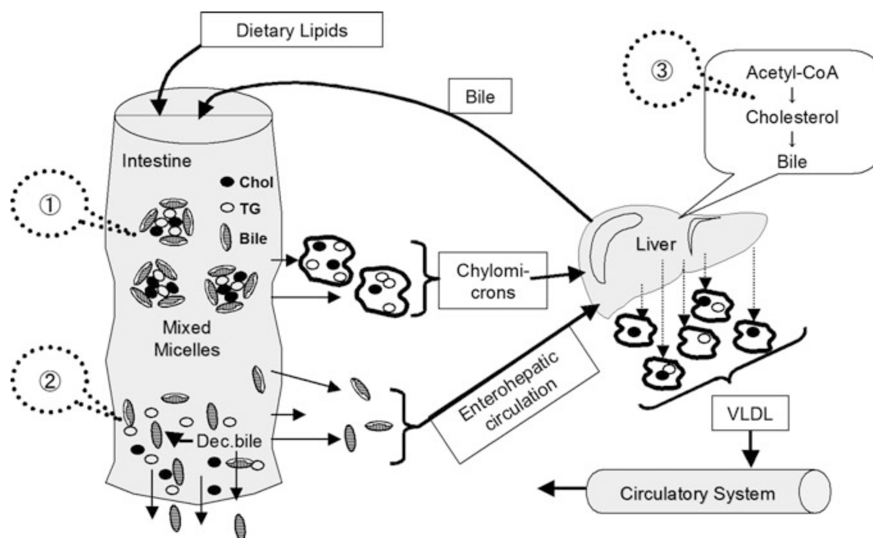


Fig. 5.4 Possible mechanisms for lowering serum cholesterol by probiotic bacteria. ①Removal of cholesterol by absorption cholesterol to the bacterial cell from the intestine. ②Deconjugation of bile salt, resulting in lower absorption in the intestinal lumen and thus more excreted in feces. ③Inhibition of cholesterol synthesis in the liver by short chain fat acids (SCFAs) generated by the bacteria (*Chol* cholesterol, *TG* triglyceride, *Dec.bile* deconjugated bile, *VLDL* very low-density lipoprotein)

resides in the mucus layer, reversed high-fat diet-induced metabolic disorders, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance. In another study, Källiomaki et al. (2008) studied stool samples that were collected from children ages 6 and 12 months, who were followed until age 7 years, to analyze the gut microbiota composition. It was found that the children who were obese at the age 7 years had fewer Bacteroidetes than the leaner children, whereas obese children at 6 and 12 months of age had more *Staphylococcus aureus* than the lean children (Kalliomäki et al. 2008). These finding strongly suggested that the composition of the gut microbiota influence the development of obesity.

Low-grade inflammation has also been shown to play a role in the development of the pathogenesis associated with obesity. It has been suggested that lipopolysaccharides (LPS) were involved in the development of the metabolic syndrome associated with high-fat intake (Cani et al. 2007, 2008, 2009). These reports described that mice fed on a high-fat diet for 2–4 weeks exhibited a significant increase in plasma LPS. This increase in plasma LPS induced by a high-fat diet was suggested to be a trigger of the development of metabolic syndrome.

Some studies have demonstrated the effect of probiotic supplementation in the management of body weight and body fat. Lee et al. demonstrated the anti-obesity effect of *L. rhamnosus* PL60, a strain producing conjugated linoleic acid, in a study

that was done on diet-induced obese mice (Lee et al. 2006). It was found that obese mice given *L. rhamnosus* had reductions in the body weight and white adipose tissue (epididymal and perirenal) (Park et al. 1997). In a human study, *L. gasseri* LG2055 was demonstrated to significantly lower the abdominal visceral and the subcutaneous fat areas, body weight, body mass index, waist and hip circumferences, and body fat mass (Kadooka et al. 2010). In addition, in a study to check the dose effect, it was demonstrated that consumption of *L. gasseri* LG2055 at doses of 10^8 cfu/d exhibited a significant lowering of abdominal adiposity and suggest that constant consumption might be needed to maintain the effect (Kadooka et al. 2013). In a study conducted by Krishan et al. (2011) to evaluate the effects of the probiotic *L. rhamnosus* on the anthropometric parameters and lipid profiles in adults, it was found that the subscapular skinfold thickness and the suprailiac skinfold thickness were significantly decreased at the end of 12 weeks of treatment. Kondo et al. (2010) reported that administration of a probiotic strain, *B. breve* B-3, in a mouse model of diet-induced obesity reduced body weight gain and visceral fat deposit in a dose-dependent manner and improved serum levels of total cholesterol, glucose, and insulin. DNA microarray analysis revealed that administration of *B. breve* B-3 regulated the expression of genes related to lipid metabolism as well as responses to stress in the liver. These results suggest that changed fatty acid metabolism and suppressed systemic inflammation in the liver contribute to the mechanisms for the anti-metabolic syndrome effects of *B. breve* B-3 (Kondo et al. 2013a).

These results suggest the potential of probiotics to influence the metabolic disorders. Modification of microbiota and suppression of low-grade inflammation were suggested as the underlying mechanisms of action. Although studies using GF mice demonstrated a close association of gut microbiota with obesity (Bäckhed et al. 2004; Ley et al. 2005), few studies have demonstrated the changes of gut microbiota by probiotic administration in obesity animal models. No effect was found by the administration of *B. breve* B-3 on the copy numbers of Bacteroidetes or the ratio of Bacteroidetes to Firmicutes in the B-3 group. Instead, they found that the fecal and cecal cell counts of *Bifidobacteria* significantly increased in the B-3 groups as compared to the control group. The increased copy numbers and the proportion of *Bifidobacteria* in the cecal and fecal microbiota was suggested to relate to the permeability of gut, intestinal endotoxin levels and mucosal barrier function, consequently suppressing the inflammatory reaction and increase in fat and body weight induced by high-fat diet, as suggested by Cani et al. (2007, 2009). Wang et al. (2013) found that probiotic *L. casei* Zhang could protect rats from LPS-induced liver injury via a NF-κB and TLR4 receptor pathway. Zhang et al. (2013) indicated that *L. casei* Zhang could ameliorate the impaired glucose tolerance in metabolic syndrome rats via enhanced osteocalcin secretion, depending on enriched vitamin K2 production by *B. fragilis*, which suggests a novel mechanism for probiotics in the anti-metabolic syndrome effect. These findings might contribute to the development of probiotic treatment of obesity.

5.1.3.6 Effect on Glycemic Control and Type 2 Diabetes

Type 2 diabetes (T2D) has been a sharp-increasing worldwide epidemic and brought threats to global health (IDF Diabetes Atlas). As mentioned above, microbiota-driven chronic low-grade inflammation play a leading role in the progression of T2D (Ouchi et al. 2011). A metagenomic study found that changes of some taxa of gut microbes are correlated with inflammatory biomarkers such as hs-CRP, IL-6, and TNF-α in impaired and diabetic glucose European women (Karlsson et al. 2013). Probiotics are known to promoting immune response and suppress inflammation (Lomax and Calder 2009). By using a high fructose-induced metabolic syndrome model, probiotic *L. reuteri* GMNL-263 had proved to modulate intestinal microflora and decrease inflammatory IL-6 and TNF-α in adipose tissue as well as enhanced PPAR-γ and GLUT4 expression in diabetic rats (Hsieh et al. 2013). Moreover, multispecies probiotic supplementation downstreamed the serum inflammatory hs-CRP levels and improved HOMA-IR scores in T2D patients (Asemi et al. 2013). However, single probiotic *L. casei* Shirota administration had no significant effect on insulin sensitivity, β-cell function, endothelial function, and inflammation markers of metabolic syndrome people (Tripolt et al. 2013).

Another important effect of probiotics on T2D is their favorable antioxidative ability. Probiotic yoghurt fermented by *L. acidophilus* La5 and *B. lactis* Bb12 could reduce fasting blood glucose and hemoglobin A1c levels in T2D patients along with improved total antioxidant status and decreased malondialdehyde levels (Ejtahed et al. 2012). This probiotic yoghurt was also confirmed to improve total cholesterol and LDL-C concentrations in T2D patients (Ejtahed et al. 2011). In addition, multispecies probiotic reported by Asemi et al. (2013) also promoted plasma GSH levels in T2D patients.

5.1.3.7 Effect on Nervous System Development and Behavior through Gut–Brain Axis

In recent years, emerging evidence from researches supports the occurrence of cross-talk between gut and brain. Several studies have shown the association between microbiota and behavior. In animals, stress during early life of rats caused modification of fecal microbiota (Bailey et al. 2011). In a human study, elderly with high frailty scores possessed lower level of *Lactobacilli*, *Bacteroides/Prevotella* and *Faecalibacterium prausnitzii* but high number of *Enterobacteriaceae* (Van Tongeren et al. 2005). Moreover, Bailey et al. (2011) found that the proportions of *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea* are correlated with stress driven IL-6 and MCP-1 secretion.

This gut-brain axis effect was further confirmed by comparison with microbiota in germ-free mice and fecal microbiota transplantation (Diaz Heijtz et al. 2011). SPF Swiss Webster mice are often with behavior deficits but the germ-free type exhibited reduced anxiety-like behavior (Neufeld et al. 2011). More interestingly,

GF Balb/c mice transplanted with Swiss microbiota behaved with lower anxiety while GF Swiss mice transplanted with Balb/c microbiota behaved enhanced anxiety (Bercik et al. 2011). Nowadays, researchers are investigating whether GF mice will exhibit a similar behavior upon transplant of microbiota of individuals with dementia, depression, or anxiety behavior.

These observations suggested the potential role of probiotics for microbiota modulation to influence gut-brain axis in health and disease. *L. paracasei* was found to significantly attenuate postinfective muscle hypercontractility, and this was accompanied by decreased expression of inflammatory mediators in the muscle layer, such as cyclooxygenase (COX)-2 (Verdú et al. 2006). Moreover, Desbonnet et al. (2010) demonstrated that maternal separation mice administrated with probiotic *B. infantis* exhibited an improved behavioral performance in the forced swim test. Also the depressed animals receiving this probiotic showed an elevated noradrenaline in the brain as well as enhanced immune responses of body. The probiotic *B. longum* NCC3001, a synonymy designated for *B. longum* BB536, was shown to normalize anxiety-like behavior and hippocampal brain-derived neurotrophic factor (BDNF) in mice with infectious colitis (Bercik et al. 2010). Furthermore, in a colitis model, the anxiolytic effect of *B. longum* NCC3001 was found to require vagal integrity, but not to involve gut immunomodulation or production of BDNF by neuronal cells (Bercik et al. 2011).

One important finding in relation to the mechanism of emotional behavior regulation by probiotic *L. rhamnosus* JB-1 was provided by Bravo et al. (2011) using a mouse model. It was revealed that the brain GABA receptors could be regulated by *L. rhamnosus* JB-1 at different brain areas including hippocampus, amygdala, and locus coeruleus. It is finally concluded that *L. rhamnosus* JB-1 have potential in relieving anxiety and depression. In a clinic study, yoghurt containing *B. lactis* CNCM I-2494 down-streamed the activity in the mid-posterior insula in healthy women as observed by functional magnetic resonance imaging (Tillisch et al. 2012). Another human study indicated that long-term ingestion of *Lactobacillus helveticus* R0052 and *B. longum* R0175 administration could lower levels of stress biomarkers in healthy people (Messaoudi et al. 2011). Recently, Tillisch et al. (2013) found that multispecies probiotic supplementation reduced task-related response including affective, viscerosensory, and somatosensory cortices in healthy women.

With regard to the potential role of gut bacteria in the development of brain, it is assumed that probiotic may alleviate nervous deficits under some medical conditions. Though *B. longum* NCC3001 had no effect on inflammation in chronic colitis mice, it could decrease excitability of enteric neurons and improve anxiety-like behavior in these mice. Additionally, Davari et al. (2013) have shown that a combined probiotics (*L. acidophilus*, *B. lactis*, and *Lactobacillus fermentum*) could efficiently reverse deteriorated brain functions in the levels of cognitive performances and their proposed synaptic mechanisms in diabetes mellitus.

5.1.4 Future Trends: Post-Probiotics and Other New Concepts and Applications

Probiotics are defined as ''live microorganisms which when administrated in adequate amounts confer a health benefit on the host.'' However, it has been reported that dead cells and, in some cases, even cell components can have positive health effects. In addition, some probiotic strains have exhibited beneficial effects without marked alternation of intestinal microbial balance, in particular for immune modulation. As a result, there is argument for the need of expanding the definition of probiotics (Salminen et al. 1998).

Fundamentally, the effects by probiotics can be derived from two aspects, direct effects via the cell components and indirect effects via modulation of intestinal microbiota. The former contributes to immunomodulatory effects, which can be obtained via interaction between the cell components and the intestinal immune system mediated by intestinal antigen presenting cells (biogenic effects); the later contributes to the generation or stabilization of a balanced gut microbiota (probiotic effects). The effects of probiotics could be thus be a dual one where live probiotic cells might well influence the gastrointestinal microbiota and have an immunomodulating effect by the cell components. On the other hand, the components of dead cells could exert an immunomodulating effect such as an anti-inflammatory response. In fact, there is considerable published evidence that bacterial dead cells can exert biological effects.

Substantial interest has been shown for the application of nonviable microorganisms in food or food supplements (Adams 2010). At first, the use of nonviable microorganisms could solve the problem concerning the stability of active constituents in handling and preservation and could prolong the shelf-life of products. Furthermore, nonviable microorganisms could eliminate the risks of microbial translocation, invasion, and toxin production (Taverniti and Guglielmetti 2011).

With the accumulative knowledgement of various species colonized in gut, there is strong evidence for increasing the probiotic list and high throughput approaches has allowed to developing new probiotics. *Faecalibacterium prausnitzii* was considered as a new probiotic due to anti-inflammatory effect which account for more than 5 % of the total bacterial population in gut (Sokol et al. 2008). Recently, *A. muciniphila*, a human intestinal mucin-degrading bacterium, was demonstrated to reverse high-fat diet-induced metabolic disorders in mice (Everard et al. 2013). Interestingly, *A. muciniphila*-increasing mechanism was involved in the therapeutic effect of commonly used anti-diabetic metformin (Shin et al. 2013). As one of most abundant genera in microbiota, strains of *Bacteroides* are primary candidates for screening potential probiotics. For example, *Bacteroides uniformis* CECT 7771 showed anti-obesity effect and improve immune defense in mice (Gauffin Cano et al. 2012). Another category of potential probiotics is equol-producing strains in gut. Up to date, novel strains are being targeted for probiotic screening, such as anaerobic SNU-Julong 732, *Adlercreutzia equolifaciens*, *Slackia equolifacien*s DZE, *Slackia isoflavoniconvertens* (Wang et al.

2005; Maruo et al. 2008; Jin et al. 2010; Matthies et al. 2012). In addition, *Oxalobacter formigenes* which could increase colonic absorption of oxalate has been confirmed to reduce the risk of stone recurrence in patients (Siener et al. 2013). Besides, *Mycobacterium vaccae* exerted a beneficial effect on behavioral performance of mice (Matthews and Jenks 2013).

5.2 Probiotic Products

5.2.1 Introduction

Lactic fermentation has traditionally been employed to preserve foods, and LAB are the main microbes involved in the natural fermentation process. During the past three decades, many strains from the species of LAB and *Bifidobacteria* have been classified as probiotics. These beneficial microorganisms are characterized with various health promoting effects such as balancing of intestinal microbiota, adjustment of intestinal immune system, inhibition of enteric pathogens, supply of antimutagens and antioxidants (Park et al. 2007). Table 5.4 shows some commercialized probiotic strains.

Probiotic products can be defined as microbial cell preparations or processed products containing sufficient quantities of viable probiotic microorganisms that have beneficial health effects on the host (Tamime 1999). There are generally two forms of probiotic products: supplements and foods. The probiotic supplements are generally freeze-dried bacterial preparations in the form of capsules, tablets, or sachets. The most frequently used foods for addition of probiotics are fermented dairy products such as yoghurt, cheese, ice cream, etc. Recently, nondairy probiotic foods have attracted increasing attention, including probiotic cereals, fruits and vegetables, meats, etc.

It is of significance to maintain sufficient quantities of viable probiotic microorganisms in the product in order to exert their beneficial health effects in the host. Generally, a minimum population of 10^6–10^7 CFU/g viable probiotic cells is required in the final products, so that a daily probiotic ingestion of 10^8–10^9 CFU can be reached when an average of 100 g or 100 mL of food is consumed per day (Jayamanne and Adams 2006).

During the last decade, there have been many kinds of probiotic products available on the market in different countries (Table 5.5). Probiotic products constitute one of the largest segments of the functional foods market. It was reported that the probiotic product market reached $27.9 billion in 2011, and was forecasted to reach $44.9 billion in 2018. Dairy products, e.g., fermented milk and drinks, have been the most popular form of consumption of probiotics, and they account for the highest market share of functional foods (Sánchez et al. 2009). Nowadays, there is continuously growing demand for probiotic products since consumers are more aware of the health benefits by consumption of these products.

Table 5.4 Examples of some commercially available probiotic strains

Strains	Brand/Origin
L. acidophilus NCFM	Rhodia, USA
L. acidophilus NCFB 1748	
L. acidophilus LA-5	Chr. Hansen, USA
L. bulgaricus LBY27	
L. casei CRL 431	
L. casei Imunitass DN-114 001	Danone, France
L. casei Shirota YIT 9029	Yakult, Japan
L. casei Zhang	Inner Mongolia Agricultural University, People's Republic of China
L. johnsonii LA1 (NCC 533)	Nestlé, Switzerland
L. paracasei F19	Arla Foods, Denmark/Sweden
L. plantarum 299 V	Probi AB, Sweden
L. plantarum P-8	Inner Mongolia Agricultural University, People's Republic of China
L. reuteri RC-14	Urex, Canada
L. rhamnosus 271	
L. rhamnosus GG (ATCC 53103)	Valio, Finland
L. rhamnosus GR-1	
B. animalis DN173 010	Danone, France
B. breve Yakult	Yakult, Japan
B. lactis Bb-12	Chr. Hansen, USA
B. lactis HN019 (DR10)	Danisco, USA
B. lactis V9	Inner Mongolia Agricultural University, People's Republic of China
Escherichia coli Nissle 1917	Mutaflor, Australia
Saccharomyces boulardii	Enterol, Biocodex, France

5.2.2 Probiotic Dairy Products

Dairy products have been considered to be an ideal vehicle for delivering probiotic microorganisms to the human gastrointestinal tract. The major components of milk such as protein and fat, and some dairy products, e.g., cheese, may provide protection for the survival of probiotics under acidic conditions (Sharp et al. 2008). Different prebiotics or plant components can also be added to different types of probiotic dairy products to improve the probiotic viability and physicochemical properties of the products (Table 5.6). At present, many dairy products with probiotic bacteria have emerged on the food market such as pasteurized milk, fermented milks, cheeses, baby feed milk powder, ice-cream, and other dairy desserts. They have shown rapid growth with increasing number of available products, and this has also contributed to increased public familiarity with probiotic concept.

Table 5.5 Examples of some commercial probiotic products

Brand/ Trade name	Product	Probiotic microorganism	Producer
Actimel	Drinking yoghurt	*L. casei* Imunitass	Danone, France
Gefilus	LGG products	*L. rhamnosus* GG	Valio Dairy, Finland
Guangming	Fermented milk	*L. acidophilus*, *L. plantarum*, *B. lactis*	Bright Dairy, People's Republic of China
Hellus	Dairy products	*L. fermentum* ME-3	Tallinna Piimatööstuse, AS, Estonia
Mengniu	Fermented milk	*L. acidophilus*, *B. lactis*	Mengniu Dairy, People's Republic of China
ProViva	Fruit drink and yoghurt	*L. plantarum*	Skåne mejerier, Sweden
Rela	yoghurts, cultured milks, and juices	*L. reuteri*	Ingman Foods, Finland
Sci-Plus	Tablet	*L.casei* Zhang, *L. plantarum* P-8, *B. lactis* V9	Sci-Plus Biotech., People's Republic of China
Yakult	Fermented milk	*L. casei* Shirota	Yakult, Japan
Yili	Fermented milk	*L. acidophilus, B. lactis*, *B. longum*	Yili Dairy, People's Republic of China
Yosa	yoghurt-like oat product	*L. acidophilus*, *B. lactis*	Bioferme, Finland
Vifit	Drinking yoghurts	*L. rhamnosus* GG	Campina, the Netherlands

5.2.2.1 Fermented Milks

Among different kinds of dairy products containing probiotic bacteria, fermented milk, especially yoghurt is the most popular form of probiotic carrier and most consumed in the world. Probiotic yoghurt is made with the starter culture composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* accompanied with probiotic bacteria. Generally, there are three methods to incorporate probiotic bacteria into fermented milk: (1) add the probiotic bacteria together with the traditional yoghurt starter cultures; (2) the probiotic bacteria and traditional starter cultures are added to separate batches of milk for fermentation, which are then mixed together; (3) use a probiotic culture alone for milk fermentation, which may take several days due to slow growth of probiotic bacteria, e.g., the manufacture of Yakult with *L. casei* strain Shirota (Tamime et al. 2011).

During the manufacture of probiotic fermented milk, it is important to maintain survival of probiotics in the fermented milk since many probiotic bacteria do not grow rapidly in milk (Zare et al. 2012). The traditional fermentation conditions (e.g. fermentation temperature at 20 or 30 °C) may be unfavorable for the growth of probiotics, i.e., particular those that stem from the human gastrointestinal tract with optimum growth temperature of 37 °C. Other factors that affect the viability of probiotics in yoghurt include acid accumulation, inoculation level, strain variation, interaction with starter cultures, level of dissolved oxygen and hydrogen

Table 5.6 Effect of prebiotics or plant components on probiotic viability and physicochemical properties of dairy products

Product	Prebiotics/Plant components	Probiotics	Effect	Reference
Yoghurt	Inulin and polydextrose	*L. paracasei*	Improved physical and sensory properties; probiotic viability maintained at a highly acceptable level during 21 days of storage	Srisuvor et al. (2013)
	Passion fruit fiber	*L. acidophilus, B. animalis* subsp. *lactis*	Increased viscosity, firmness, consistency, and cohesiveness; more compact casein gel; improved fatty acid profile; no negative effect on sensory properties	Do Espírito Santo et al. (2012a, b, 2013)
	Soy and pulse ingredients	*L. acidophilus, L.rhamnosus*	Significantly enhanced acidification rate; enhanced growth of lactobacilli	Zare et al. (2012)
	Inulin	*L. acidophilus, L.rhamnosus, B. animalis* subsp. *lactis*	Improved firmness; increased viable cell counts; increased acidification rate	Oliveira et al. (2009, 2011)
	Green tea extract	*L. acidophilus, B. animalis* subsp. *lactis*	Increased viable probiotic counts, but decreased counts of potential pathogens in feces of healthy adults	Savard et al. (2011)
	Inulin	*L. reuteri, L.rhamnosus*	Significantly increased probiotic survival with better survival of *L.rhamnosus* than *L. reuteri*	Hekmat et al. (2009)
	Citrus fibers	*L. acidophilus, L. casei, B. bifidum*	Increased survival of *L. acidophilus* and *L. casei*, but not *B. bifidum*	Sendra et al. (2008)
	Fructooligosaccharides and inulin	*L. casei, B. lactis*	No significant effect on probiotic growth; increased nutritional quality by increasing levels of free fatty acids and conjugated linoleic acid in the product	Rodrigues et al. (2011)

(continued)

Table 5.6 (continued)

Product	Prebiotics/Plant components	Probiotics	Effect	Reference
Cheese	Fava bean starch	*L. rhamnosus, B. breve*	No significant effect on probiotic viability in Panela cheese; softer cheese texture but no effect on perceived taste or appearance	Escobar et al. (2012)
	Inulin	*L. delbrueckii*	No effect on probiotic counts in cottage cheese; no alteration in physicochemical properties	Araújo et al. (2010)
	Inulin and oligofructose	*L. acidophilus, B. animalis* subsp. *lactis*	Increased probiotic viable counts; Improved sensory quality of petit-suisse cheese	Cardarelli et al. (2008)
Frozen product	Inulin	*L. acidophilus*	Positive effect on probiotic survival; improvement of nutritional and functional value of guava mousses; improvement of texture profile of the product with reduced fat	Buriti et al. (2010a, b)
	Resistant starch	*L. casei, B. animalis* subsp. *lactis*	Positive effect on the sensory properties of ice cream with encapsulated probiotic bacteria	Homayouni et al. (2008)
Yoghurt drink	Corn fiber, polydextrose, chicory inulin	*L. acidophilus, B. animalis* subsp. *lactis*	No effect on probiotic viability; alteration of sensory properties	Allgeyer et al. (2010)
Lactic beverage	Oligofructose	*L. acidophilus, B. animalis* subsp. *lactis*	No significant effect on probiotic viability and technological properties of the product	De Castro et al. (2009)

peroxide, nutrient composition of yoghurt, and storage condition (N'guessan et al. 2011). It is known that different bacterial species and strains vary widely in their response to stress conditions. For example, *B. longum* tolerated poorly to temperature increase, oxygen, or desiccation compared to *B. animalis* subsp. *lactis* (Simpson et al. 2005). An excessively high inoculated level of *L. acidophilus* resulted in decreased *Lactobacillus* counts in yoghurt (Olson and Aryana 2008). Survival of probiotic *L. acidophilus* was influenced by the low pH of the environment due to addition of fruit mixtures or the decreased post storage pH in yoghurt (Kailasapathy et al. 2008). Stirred-yoghurt may not be a suitable food matrix for probiotic bacteria due to incorporation of oxygen into yoghurt during stirring. However, yoghurt starter culture bacteria that are also identified as oxygen scavengers may help the probiotic survival in yoghurt since they may utilize most of the oxygen in milk during fermentation to minimize the oxygen effect on the probiotics (Dave and Shah 1997).

The probiotic viability in fermented milk can be enhanced by using different methods including supplementation with prebiotics and nutrients, cell immobilization, and use of gases to eliminate oxygen toxicity, etc. Oliveira et al. (2009, 2011) reported that inulin used in the fermentation of skim milk exhibited remarkable prebiotic effect and the firmness of the fermented milk was improved when using pure cultures of probiotic *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, and *B. lactis*, or the binary co-cultures of them with *S. thermophilus*, or the mixed culture of all these strains. The use of green tea extract in yoghurt containing *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* LA-5 significantly increased the viability of the probiotics (Savard et al. 2011). The probiotic viability can be enhanced by immobilization of the probiotic bacteria in various supports such as starch, fruit pieces, casein, and wheat grains, probably due to formation of protective microenvironment (Sidira et al. 2013). Fermentation with milk gassed with nitrogen and/or hydrogen significantly increased the survival of probiotic *B. bifidum* in the fermented milk products (Ebel et al. 2011).

Probiotic fermented milks can be produced by addition of probiotics to different types of milk to meet diverse needs of the consumers. Goat's milk can be used as an alternative for cow's milk to produce dairy products due to fewer allergic reactions (Uysal-Pala et al. 2006). However, the fermented milk made with goat's milk generally gives poorer consistency than cow's milk due to the difference in casein contents and its composition (Guo 2003). Wang et al. (2012a) developed a probiotic goat's milk containing *L. acidophilus*, *L. casei*, and *Bifidobacterium* spp. with polymerized whey protein and pectin as gelling agents. These probiotic strains survived well during storage at 4 °C except *L. acidophilus* that had no viable counts by the fourth week. The use of polymerized whey protein improved the consistency of goat's milk yoghurt. Ranadheera et al. (2012) reported the preparation of probiotic plain and stirred fruit yoghurts from goat's milk using a mixed probiotic culture comprising *L. acidophilus* LA5, *B. animalis* subsp. *lactis* Bb-12 and *Propionibacterium jensenii* 702. All these probiotic strains survived well throughout the storage period at 4 °C for 4 weeks; addition of fruit juice appeared to support the viability of lactobacilli.

Probiotics can also be added to milk to produce fermented milk with special functions. The presence of probiotic *L. acidophilus* NRRL B-4495, *L. reuteri* B-14171, *L. rhamnosus* NRRL B-442, *L. johnsonii* B-2178 and *B. bifidum* B-41410 in milk reduced the bioaccessibility of aflatoxin M1, a potential strong carcinogenic compound (Serrano-Niño et al. 2013). Supplementation with micronutrients such as various vitamins, iron, selenium, zinc, and DHA, etc., in probiotic fermented milk with *L. rhamnosus* CAN-1 may provide beneficial effects for nutrition and immune function for people living with Human Immunodeficiency Virus (HIV) (Hemsworth et al. 2011). Carrot juice can be added to probiotic fermented milk as a carrier of vitamins, minerals, dietary fiber, flavonoids, and carotenoids that are beneficial in preventing diseases (Cliff et al. 2013). Fermented milk containing probiotic *L. helveticus* IDCC3801 was found to improve amyloid precursor protein metabolism in Alzheimer's disease and memory deficit (Yeon et al. 2010).

The use of some probiotics in fermented milks may negatively influence the sensory and textural characteristics of the products because they usually grow slowly in milk due to the lack of proteolytic enzymes (Dave and Shah 1998). Production of acetic acid (acetic acid/lactic acid = 3:2) during growth of bifidobacteria may result in off-flavor of the product with vinegar-like taste. One way to improve the sensory quality of probiotic fermented milk is by fortification with different milk proteins. Akalin et al. (2012) reported that fortification of probiotic yoghurts with sodium calcium caseinate (SCaCN), whey protein concentrate (WPC) or a blend of them had different effects: fortification with SCaCN improved the firmness and adhesiveness; WPC enhanced water-holding capacity more than the caseinate, and gave finer and better structures than the latter. Fortification of milk base with WPC, sodium caseinate, and skimmed milk powder to the same level of protein content improved the rheological properties of probiotic yoghurt, and partial replacement of skimmed milk powder with WPC and sodium caseinate further enhanced the rheological properties of the yoghurt (Marafon et al. 2011a, b).

The presence of viable probiotic bacteria is important to assure the beneficial health effects of the probiotics for the host, since the physiological functionality of fermented dairy foods is inherent to the intrinsic biological activity of the microorganisms (Cruz et al. 2009a). It is important to monitor the survival of the probiotics during processing and storage of fermented milk, and several methods can be used, including conventional microbiological enumeration and different molecular methods. The viability of *L. casei* ATCC 393 in probiotic yoghurts was effectively monitored by the multiplex PCR method that could rapidly detect and identify the target microorganism (Sidira et al. 2013). The shelf-life of probiotic flavored yoghurt can be predicted by a survival analysis methodology involving microbiological and sensory analyses (Cruz et al. 2010).

During the last decade, probiotic microorganisms have been incorporated into different types of traditional fermented milks to enhance their health benefits (Farnworth 2003). These include: (1) Nordic-cultured buttermilk made by fermentation of pasteurized skim or whole milk. Fermentation is done with mesophilic LAB at 20 °C for ~20 h (final pH is 4.5–4.6), followed by stirring, cooling,

flavoring (optional), and packaging. (2) Kefir traditionally made by fermentation at 18–25 °C with kefir grains (2–10 g/100 mL) for 18–24 h, followed by stirring and cooling. (3) Viili, a traditional fermented milk product in Finland. Fermentation is done at 20 °C for 20 h (final pH ~ 4.3) with mesophilic starter cultures together with a mold (*Geotrichum candidum*). A probiotic viili product containing *L. rhamnosus* GG has been developed and available on the market. (4) Greek-style yoghurt, a traditional fermented milk product in the Middle East. A Greek-style yoghurt product made with ultrafiltered milk contained bifidobacteria with viable counts between 2×10^5 and 4×10^7 CFU/g (Mahdi et al. 1990).

5.2.2.2 Cheese Products

After a successful use of probiotics in fermented milk during the 1990's, there has been an increasing interest in incorporation of probiotics into cheese, which provides a valuable alternative to fermented milk as a food carrier for probiotics with certain potential advantages. For example, the pH of cheese matrix is generally higher, creating better environment than fermented milk for probiotic survival. The closed and dense matrix, and the relatively high-fat content of cheese may serve as a protective factor for probiotics during both the food storage and the passage throughout the gastrointestinal tract. Cheese also generates a buffer effect that is favorable for probiotic survival under acidic conditions in the gastrointestinal tract (Karimi et al. 2012). A whey cheese matrix was shown to protect the probiotic strains of *L. casei*, *L. acidophilus* and *B. animalis* upon their exposure in simulated gastrointestinal conditions (Madureira et al. 2011). During the last decade, there has been a wide variety of cheeses incorporated with probiotic bacteria, such as Cheddar, Gouda, Edam, Emmental, Domiati, Cottage, Crescenza, Ras soft Herrgård, Quarg, Argentinian fresh, Egyptian Kariesh, white-brined, Minas fresh, petit-suisse cheese (Tamime et al. 2011; Vinderola et al. 2009).

To select probiotic bacteria suitable for cheese production, it is important that these microorganisms are able to retain their viability during cheese processing, and survive the long cheese ripening and storage time. The incorporation of probiotic bacteria should not markedly affect the sensory profile of cheese due to increasing accumulation of microbial metabolism, leading to undesirable changes with respect to their flavor, aroma, texture, and other important attributes. Additional features include sensitivity to oxygen, rate of acid production, and ability of growth in milk-based media (Talwalkar and Kailasapathy 2004). Furthermore, the probiotic bacteria incorporated in cheese must be able to survive during passage through the gastrointestinal tract, and thus beneficially affect the host health (Stanton et al. 2003).

During probiotic cheese production, there are several methods to introduce probiotic microorganisms into cheeses (Tamime et al. 2011). The probiotic cultures can be added to milk as adjunct cultures together with the cheese starter cultures prior to cheesemaking. Care must be taken with this method since large numbers of the probiotic cells may be lost in the whey during whey draining,

especially for slow-growing probiotic bacteria. Another method of introduction is done by addition of a dried probiotic culture during salting of curd to minimize the losses of bacterial cells to whey, especially for making probiotic semi-hard and hard cheeses. For some cheese varieties, such as cottage cheese, probiotic cultures can be incorporated together with fermented cream dressings, and some probiotic organisms such as *L. rhamnosus* GG and *B. infantis* have been successfully introduced to cheese with high viable counts in the products.

Application of probiotics in cheese represents a challenge due to technological hurdles unfavorable for their survival in the cheese processing. Various compositional and process factors obviously affect the survival of probiotic bacteria in cheese, including strains of probiotic cultures, pH, titratable acidity, hydrogen peroxide, bacteriocins, flavoring agents, microbial competitions, packaging materials, rate of inoculation, concentration of metabolites such as lactic and acetic acids, dissolved oxygen, redox potential, supplementation of milk with nutrients, incubation temperature, storage temperature, addition of salt, and antimicrobial preservatives (Karimi et al. 2012). In addition, viability of these probiotic microorganisms as adjunct cultures may also be affected by the cheese starters including LAB, mold or yeasts that may be antagonistic, competitive, or symbiotic toward each other. The survival of microorganisms can also be affected by the level of salt. When the salt concentration in cheese was higher than 4 %, the viability of probiotic bacteria was significantly decreased (Gobbetti et al. 1998). Therefore, processing of cheeses with high salt content should be optimized to facilitate incorporation of probiotic bacteria. It is known that most members of probiotics are sensitive to oxygen due to decreased oxygen-scavenging ability or complete absence of the oxygen-scavenging system in these microorganisms. Accumulation of toxic oxygen metabolites such as superoxide anion (O^{2-}), hydroxyl radical (OH^{-}), and hydrogen peroxide (H_2O_2) in the probiotic cells after their exposure to dissolved oxygen during manufacture and storage, eventually leads to their death. Therefore, probiotic cheese products should be vacuum-packed or packed with plastic films of low oxygen permeability. During the long period of cheese ripening, the complex biochemical changes, decrease in water activity, or pH in cheese matrix may be unfavorable for the survival of probiotics. Improper handling of cheese during storage or sale, e.g., at a high temperature, may negatively affect the probiotic survival, and cause undesirable changes in cheese texture, color, and flavor that lead to consumer rejection to the product (Cruz et al. 2009a). For the Argentinian fresh cheeses added with probiotic *L. paracasei* A13, the probiotic counts increased by about one log order during storage of the product at 5 °C without negatively affecting the sensory properties of the product, but a higher storage temperature (12 °C) resulted in poorer sensory quality though a higher growth of the strain was observed (Vinderola et al. 2009).

By combination with prebiotic ingredients, which can contribute to their protection while in the food matrix, the viability of probiotics in cheese may be maintained or even enhanced (Table 5.6). These nondigestible dietary components can stimulate proliferation and activity of desirable bacteria in situ after they reach the colon in essentially intact form (Mattila-Sandholm et al. 2002). The combined

use of probiotics and prebiotics to produce symbiotic cheeses may result in the products with enhanced health benefits due to their synergistic effects over either probiotics or prebiotics alone (Maukonen et al. 2008). However, the use of fructooligosaccharides and inulin did not significantly affect the growth or viability of probiotics in curdled milk, but a synbiotic effect was apparent in terms of gradual increase in the formation of nonprotein nitrogen resulted from increased secondary proteolysis throughout the 60 days ripening time (Rodrigues et al. 2011). In addition to the use of prebiotics, there are other strategies for enhancing viability of probiotics, including selection of strains with good tolerance (to acid, bile, oxygen, etc.), microencapsulation to protect the strains, supplementation with micronutrients, exposure to sublethal levels of a given stress (low temperature, low pH, starvation, etc.) (Roy 2005; Boylston et al. 2004).

5.2.2.3 Dairy Beverages

Dairy beverages are produced from milk or its derivatives, with or without the addition of other ingredients, in which the dairy base represents at least 51 % (vol/vol) of the formulation, and can be submitted to a fermentation process using yoghurt cultures (Castro et al. 2013b). These refreshing drinking products generally have a smooth texture with low viscosity, and can serve as important carriers for probiotics. Some dairy beverage products have been developed using mixtures of whey, milk, fruit juice, probiotic bacteria, and/or yeast (Almeida et al. 2009). The inclusion of whey into probiotic dairy beverages is of significance considering value-added use of this by-product from cheese manufacturing.

The viability of probiotics in dairy beverage is dependent on the specific strain, and its interaction with other strains of the starter cultures used, as well as variation in the food matrix of the product. Elizaquível et al. (2011) evaluated various beverages and yoghurt as carriers of the probiotic lactic acid bacterial strains isolated from ciders, and found that the growth of *Pediococccus parvulus* and *L. suebicus* strains decreased by 2–3 log orders of magnitude in yoghurt during 28 days of cold storage; but there was no significant decrease of the probiotic survival in the juice and juice-milk beverages under the same storage conditions, though the viability of *L. suebicus* decreased by 3 log orders of magnitude; milk beverages and yoghurt also showed a protective effect for *P. parvulus* against gastrointestinal stress. A probiotic strain of *L. acidophilus* was shown to grow well in a liquid whey based by-product from Ricotta cheese manufacturing, and grow substantially better when combined with the use of the yoghurt starter culture strains *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in the fermented product (Maragkoudakis et al. 2010). Combination of dairy beverages processed with different levels of whey in their formulation exhibited good potential as a food matrix for supplementation with probiotics (Castro et al. 2013a). As shown by Almeida et al. (2009) that the viability of probiotic *L. acidophilus* and *B. lactis* was influenced by the total solid content in a milk-whey based beverage, and co-culture with *S. thermophilus* showed good potential to be employed in

manufacturing the product with total solids of 8 and 10 g/100 g. However, addition of prebiotic oligofructose to dairy beverages did not show any significant influence on the viability of probiotic *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* Bb-12, whereas addition of cheese whey only influenced the syneresis index of the beverages (De Castro et al. 2009). Incorporation of the prebiotics in a probiotic yoghurt drink with different levels of inulin, soluble corn fiber, and polydextrose were shown to alter the sensory properties of the product, and the viability of the probiotic *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* LA-5 showed a 2- to 3-log decrease, with or without prebiotics, after 30d of refrigerated storage (Allgeyer et al. 2010).

There are challenges related to developing dairy beverages in terms of obtaining a product with suitable rheological properties, functionality and sensory quality, which vary considerably with the formulation or ingredients of the product, as well as the processing techniques used. Castro et al. (2013b) reported that whey content in dairy beverage significantly affected the consumer acceptance of the product; increasing the amount of whey added to probiotic dairy beverage decreased the gel strength; beverages with whey contents more than 65 % resulted in lower acceptance by consumers, but higher sensory acceptance was obtained at a whey concentration of 49 %. Treatment of a probiotic beverage containing skim milk and whey with ultra-high pressure homogenization resulted in improved rheological properties, i.e., the consistency of the product, thus decreasing the need for additives and stabilizers (Masson et al. 2011). The acidifying rates of the probiotic bacteria in Minas frescal cheese whey could also be affected by the co-culture composition and the pH level at which the fermentation was stopped; co-culturing of the probiotic *B. animalis* subsp. *lactis* with *S. thermophilus* could shorten the fermentation time when the pH reached 4.5 (Almeida et al. 2008). A functional beverage could be developed by fermentation with kefir grains of whey from the manufacture of Bulgarian brine cheese and ultrafiltrate from milk and whey, using a noncontact method of process monitoring (Balabanova and Panayotov 2011). However, a probiotic whey beverage, which was manufactured with lactic culture *S. thermophilus* TA040 and *L. bulgaricus* LB340 and probiotic culture *L. acidophilus* LA 14 and *B. longum* BL 05, was found to give lower immune-protection activity than a probiotic yoghurt made with the same starter cultures in terms of blood-cell indicators (neutrophils and lymphocytes), cytokines (TNF-α and IL-1β) and other standard health parameters (Lollo et al. 2013). Due to increased demands for probiotic fermented drinks/beverages worldwide, a long shelf-life fermented milk drink has been developed by using a specially designed two-jacketed straw attached with 10^8 CFU of a freeze-dried *L. reuteri* culture with a shelf-life of 12 months at 25 °C; when the consumer drinks 100 mL of the beverage, 99 % of the probiotic bacteria are released (Tamime et al. 2011).

5.2.2.4 Ice-cream and Frozen Dairy Desserts

Ice cream and frozen dairy desserts may serve as potential food carriers for probiotics. For the nonfermented type ice cream, the probiotic microorganisms can be incorporated by direct inoculation of the mix, while the fermented type can be produced by blending the acidified milk or probiotic yoghurt with the ice cream mix. Ice cream is considered as a good vehicle for delivering probiotics in human diet since the product is generally stored under frozen conditions that may facilitate maintaining stability and viability of the probiotics. The neutral pH condition of nonfermented ice cream may also provide possibility of satisfactory survival of probiotic bacteria. Production of ice cream includes multisteps of processing, specially involving freezing and thawing operations that may damage cells, and mechanical stresses of mixing and incorporation of oxygen into the mixture that may result in lower probiotic viability, thus resulting in decreased functionality of the product (Ranadheera et al. 2010). Therefore, it is important to optimize the technical parameters of each stage during ice cream manufacturing in order to increase the probiotic viability and guarantee the product functional properties. In addition, the overall quality of ice cream should not be affected due to incorporation of probiotics, and should be comparable with conventional ice-cream regarding the physical-chemical properties of the product (Cruz et al. 2009b).

Survival of probiotics in frozen dairy desserts can be affected by high redox value, oxygen toxicity, whipping-freezing treatment, and acidity of the matrix (Cruz et al. 2009a). Selection of stable probiotic strains is of significance to assure enough viability of the probiotics in the frozen products. One way to improve the probiotic survival in the hostile environment and gastrointestinal tract is by physical protection of probiotics via microencapsulation of the bacterial cells. Encapsulation of probiotic *Bifidobacterium* spp. with alginate was shown to lead better survival of the probiotics in frozen ice milk than those with κ-carrageenan (Kebary et al. 1998), and the survival of some probiotic bacteria in fermented frozen desserts also improved with encapsulation (Shah and Ravula 2000a). In addition, selection of an adequate food matrix is of importance for enhancing probiotic survival in the specific food matrix and in the gastrointestinal tract (Schillinger et al. 2005). The use of prebiotics, e.g., inulin-type fructans were found to protect bacterial cells, and improve the survival and activity of probiotic bacteria in the food matrix (Donkor et al. 2007). Akalin and Erisir (2008) also showed that addition of inulin and oligofructose resulted in firmer ice cream during storage, and increased the survival of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* Bb-12, mainly for ice-cream containing oligofructose as functional ingredient. Whey protein concentrate may also act similarly as prebiotics to protect effectively the bacterial cells, and increase the maintenance of probiotic survival during the shelf-life (Akalın et al. 2007). The use of dairy-based components such as proteins, fat, lactose, and other components, and storage of the product at a lower temperature may help the survival of probiotics during shelf-life (Cruz et al. 2009a). Addition of different types of sweeteners, sucrose or aspartame, did not cause undesirable effects on the survival of three highly resistant (to acid and bile)

probiotic strains (*L. acidophilus*, *L. agilis* and *L. rhamnosus*), and their concentrations remained constant during 6 months storage (Başyiğit et al. 2006). Ice cream made from different levels of fat and sugar showed different survival rates for the probiotic strain *L. johnsonii* La 1 during 30 days of storage at −28 °C (Alamprese et al. 2002).

5.2.2.5 Dried Products

Probiotics can be applied for production in dried form to extend their shelf life, and powdered probiotic products have been widely used in the manufacture of dairy products. Among different drying methods for probiotics, freeze-drying is most commonly used to maintain high level of probiotic viability. Other drying methods such as freeze-, spray-, vacuum-, and fluidized bed-drying have also been increasingly used to obtain acceptable level of probiotic survival with relatively low operating costs and higher throughput capacity.

Production of dried probiotics consists of several different steps, starting with fermentation that requires appropriate ingredients, and growth conditions to be optimized with right oxygen tension, pH, and temperature (Possemiers et al. 2010). Subsequently, the probiotic biomass obtained from fermentation needs to be concentrated by centrifugation or membrane filtration. The concentrated biomass is then subjected to the drying step that involves different drying methods as mentioned. After proper residual moisture content is reached, the material is milled and blended to obtain maximized homogeneity for final packaging. Packaging materials with low oxygen and moisture transfer should be used to ensure required shelf-life of the probiotic product.

Maintaining viability of probiotics during processing and storage in dried product matrix is a challenge since their survival may be affected by different factors such as removal of water, exposure to oxygen, physical characteristics of product matrix and storage conditions, and more loss of probiotic viability may occur during storage than during processing (Jankovic et al. 2010). The stability of probiotics can be improved by using protective agents or microencapsulation of probiotics, and storing at low a_w. The most frequently used protective agent is skimmed milk. The probiotic bacteria showed better survival in the presence of milk than in single ingredients or a defined mixture of ingredients during drying and storage (Li et al. 2011; Pyar and Peh 2011). Other commonly used protective agents include sugars such as mono-, di-, or oligosaccharides, and sugar derivatives such as sorbitol (Li et al. 2011). Proteins, amino acids, and prebiotics/fibers have also been shown to improve the drying- and storage-stability of LAB (Pyar and Peh 2011; Heidebach et al. 2010). Alternatively, microencapsulation may serve as another effective strategy for bacterial cell protection against adverse conditions, and help improve probiotic stability and viability during processing and storage conditions. Reconstituted skim milk can be used as encapsulating agent to improve probiotic survival during spray drying process (Fu and Chen

2011). The encapsulated *L. acidophilus* MJLA1 survived better than free cells after 12 weeks of storage in fermented frozen desserts (Shah and Ravula 2000b).

Incorporation of probiotics in dried preparations may improve the viability of probiotic organisms in the gut, and these preparations have been used for the manufacture of infant formulae. For example, the freeze-dried preparations containing *L. acidophilus, L. reuteri*, and *B. bifidum* have been used to develop an infant formula to prevent diarrhea when consumption of the three organisms was 10^8–10^{10} CFU/day. Milk powder containing *B. animalis* subsp. *lactis* Bb-12 has also been developed for older infants (Tamime et al. 2011).

5.2.3 Nondairy Probiotic Products

Although dairy products are still the most common vehicles for delivery of probiotics to humans, the increasing consumer demands for health products have stimulated exploring various nondairy products, including fruits, vegetables, cereals, legumes, and meat. These products may provide alternative forms of probiotics for the increasing numbers of vegetarians, for consumers with lactose intolerance or dyslipidemia and those who prefer low cholesterol diets. Among probiotic microorganisms, lactobacilli and bifidobacteria are most commonly used by the nondairy probiotic food industry due to their high viability as well as good technological properties.

5.2.3.1 Probiotic Fruit and Vegetable Products

Fruits and vegetables are rich in nutrients with taste profiles appealing to all individuals and widely accepted among consumers as healthy and refreshing foods (Sheehan et al. 2007). By modifying their food structures and components, fruits, and vegetables can serve as ideal carriers for probiotics, because they contain beneficial nutrients such as minerals, dietary fiber, vitamins, and antioxidants (Betoret et al. 2012). Similarly to other plants, the fruit and vegetable tissues contain complex microstructures with intricate internal cells, intercellular spaces, pores, and capillaries, but the intact plant cell wall having rather small pore sizes (0.1–5.0 μm) may function as physical barriers against the action of microorganisms (Martins et al. 2013). Peeling and cutting, as in the cases of minimally processing fruits and vegetables, may accelerate the release of cellular content, which is rich in sugars, minerals, vitamins, and other nutrients, and these beneficial nutrients are very favorable for microbial growth (De Azeredo et al. 2011). In addition, the fermentation of plant-based materials (e.g., artichokes and table olives) with plant-originated probiotic strains results in similar survival rates of the probiotic bacteria to those of milk-originated probiotics (Ranadheera et al. 2010). Therefore, processed or fermented fruits and vegetables may provide good substrates for probiotic growth. The surface microarchitecture of plant contains ridges

and natural prebiotic oligosaccharides that may help protect probiotics against the acidic stress of the stomach, and play a positive role in maintaining bacterial survival (Ranadheera et al. 2010). Recently, an increasing interest in developing fruit and vegetable-based functional products was observed using probiotics in fruit juices, minimally processed fruit, fruit smoothies, fermented vegetables, snack products, and olive products (Table 5.7).

The viability of probiotics in fruit and vegetable juices may be affected by the pH and levels of organic acids, and the presence of total phenol and oxygen. The low pH (<3.2) and the high total phenol concentration (>1.5 g/L) of strawberry, pomegranate, and cranberry juices may cause the death of *L. plantarum* and *B. longum* during refrigerated storage of the products (Nualkaekul et al. 2011). However, *Lactococcus lactis* ssp. *cremoris* was shown to grow well in cucumber and water melon juices supplemented with ρ-aminobenzoate-1 and glutamate and also to increase the folate levels in the juice products (Gangadharan and Nampoothiri 2011). Six potential probiotic strains of *L. rhamnosus* and *L. casei* were able to grow and to survive at a population level higher than 10^8 CFU/mL in chestnut puree along 40 days of storage period at 4 °C (Blaiotta et al. 2012). Pereira et al. (2011) optimized the cultivation conditions of the probiotic strain of *L. casei* NRRL B442 in cashew apple juice to be initial pH 6.4, inoculation level of 7.48 Log CFU/mL, fermentation temperature of 30 °C and fermentation time of 16 h, and the results showed that the viable cell counts were higher than 8 Log CFU/mL throughout 42 days of the refrigerated storage period. However, the growth of *L. casei* NRRL B442 in sonicated pineapple juice resulted in lower viable counts, i.e., 6.03 Log CFU/mL in the nonsweetened sample and 4.77 Log CFU/mL in the sweetened sample, after 42 days of refrigerated storage (Costa et al. 2013). *B. lactis* Bb-12 and *B. bifidum* B7.1 and B3.2 could grow well in pure carrot juice after 6 h of incubation with probiotic count of 10^8 CFU/mL, carotenoid degradation rate between 15–45 %; production of lactic and acetic acids in the range of 14.8–16.7 mg/mL and 3.3–5.3 mg/mL, respectively (Kun et al. 2008).

Foods of plant origins such as fruits and vegetables generally contain high amount of dietary fibers that are nonstarch polysaccharides, including cellulose, pectin, hemicelluloses, β-glucans, lignin, and gums. Some of the dietary fibers may function as prebiotics that may promote the survival of probiotic bacteria during passage through the gastrointestinal tract, beneficially affecting the health of the host. The high concentration of dietary fibers in the fruit juices such as apple, orange, blackcurrant, grapefruit, pineapple, and lemon may be beneficial to the survival of probiotics during refrigerated storage (Nualkaekul et al. 2012b). Moreover, some fibers may also protect the probiotics during freeze-drying and storage. The use of oat bran with 9 % β-glucan and green banana flour during vacuum drying of *L. casei* LC-1 increased substantially the survival of the probiotics, and the bacterial cells were shown to adhere well to fibers without morphological changes after drying (Guergoletto et al. 2010). Addition of oat flour containing 20 % β-glucan in apple juice (pH $\sim$ 3.5) protected *L. rhamnosus* during storage at 20 °C (Saarela et al. 2006). Addition of oat fiber to mixed fruit juice improved the stability of *B. breve* during refrigerated storage (Saarela et al. 2011).

Table 5.7 Recent studies on the use of probiotics in fruit and vegetable products

Product	Probiotics	Findings	Reference
Dried apple slices	*L. rhamnosus*	Good survival of the probiotics for 30 days at 25 °C or 180 days at 4 °C	Noorbakhsh et al. (2013)
Low humid apple snack	*L. salivarius* ssp. *salivarius*	Good survival of the probiotics in the product with potential effect against *Helicobacter pylori* in children	Betoret et al. (2012)
Instant fruit powders	*L. plantarum*	Best probiotics survival in black currant powder, followed by strawberry, pomegranate, and cranberry powder	Nualkaekul et al. (2012a)
Fermented green table olives	*L. pentosus*	Reduced growth of different pathogens in the product fermented with the probiotics	Argyri et al. (2013)
Carrot juice	*B. bifidum, B. animalis* subsp. *lactis, L. casei*	Good growth of the *Bifidobacterium* strains without any nutrient supplementation	Kun et al. (2008)
Fermented chestnut purees	*L. casei, L. rhamnosus*	Good survival of the probiotics with counts of over 8 Log CFU/mL at 4 °C for 40 days	Blaiotta et al. (2012)
Pineapple juice	*L. casei*	Good viability of the probiotics; no change in the characteristic color; no browning	Costa et al. (2013)
Cashew apple juice	*L. casei*	Good survival with probiotic counts of higher than 8 Log CFU/mL at 4 °C for 42 days	Pereira et al. (2011)
Fresh-cut apple slices and papaya products	*L. rhamnosus, B. animalis* subsp. *lactis*	Good survival with probiotic counts of higher than 8 Log CFU/mL at 2–4 °C for 14 days; good adherence of the probiotics to the surface of apple wedges	Alegre et al. (2011), Rößle et al. (2010a, b)
Fruit cocktail of carrot, celery and apple	*L. acidophilus*	Good survival (6–7 Log CFU/g) of the probiotics in the juice mixture without supplementation of other nutrients	Nicolesco and Buruleanu (2010)
Pomegranate juice	*L. plantarum, L. delbrueckii, L. acidophilus, L. paracasei*	*L. plantarum* and *L. delbruekii* showed higher viability during the first 2-week storage but decreased dramatically after 4 weeks; citric acid as a major organic acid in the juice was significantly consumed by all the probiotics	Mousavi et al. (2010)

(continued)

Table 5.7 (continued)

Product	Probiotics	Findings	Reference
Fermented pear juice	*L. acidophilus*	Total phenolics decreased with fermentation and DPPH linked antioxidant activity increased; α-Glucosidase inhibitory activity significantly increased for fermented acidic samples	Ankolekar et al. (2012)
Fermented barley, whey powder and tomato pulp mixtures	*L. acidophilus*	Good survival (>8 Log CFU/g) of the probiotics; feeding of fermented mixture containing the probiotics was beneficial in controlling *E. coli* induced diarrhoea in mice	Jood et al. (2012)

Vacuum impregnation can be used to introduce probiotics into the porous structure of fruits and vegetables that have intercellular spaces filled with gases and liquid. The gas and liquid can be removed by vacuum and replaced by diffusion with the probiotics. This technique has been successfully used in apples that contain relatively high percentage of intercellular spaces in parenchyma, being 20–25 % of the total volume (Aguilera 1999). Noorbakhsh et al. (Noorbakhsh et al. 2013) introduced probiotic *L. rhamnosus* ATCC 7469 into apple slices by vacuum impregnation, and the probiotic-enriched apple slices were subsequently dried by air drying, freeze drying, and a combination of air drying and radiant energy vacuum (REV) drying. The samples treated with air drying+REV drying had the longest shelf- life during storage at 25 °C, followed by freeze drying and air drying; the dried apple slices were also found to protect the cells in acidic gastric juice. Betoret et al. (2012) developed a probiotic low humid apple snack containing a probiotic strain of *Lactobacillus salivarius* spp. *salivarius* (about 10^8 CFU/g) that was introduced by vacuum impregnation, and the product was tested to be potentially effective against infection caused by *Helicobacter pylori*. Good survival of probiotics can also be achieved by preparing dried fruit powder/probiotic mixtures. Nualkaekul et al. (2012a) showed good survival of the freeze-dried *L. plantarum* cells when mixed with freeze-dried strawberry, pomegranate, blackcurrant, or cranberry powder during storage, and in the reconstituted fruit juices at room temperature for up to 4 h, indicating that instant juice powders can serve as good probiotic carriers.

Table olive has been considered as a good vehicle to deliver probiotics to humans due to its microarchitecture and the presence of nutrients. Diverse groups of microorganisms are involved in the fermentation of table olives, including Enterobacteriaceae, Propionibacteriaceae, LAB, and yeasts. Among these microorganisms, LAB play beneficial roles, e.g., in degradating oleuropein, thus resulting in debittering of the fruits. Recently, the probiotic bacterium *L. paracasei* IMPC2.1 carried by olives has been shown to colonize the gut of healthy and

constipated subjects (Lavermicocca 2006; Sarvan et al. 2013), and application of this probiotic bacterium in table olives or artichokes has been successfully commercialized in Italy (Lavermicocca et al. 2003). Bellis et al. (2010) showed that *L. paracasei* IMPC2.1 played dual roles as starter and probiotic cultures during fermentation of table olives; *L. paracasei* IMPC2.1 could colonize the olive surface dominating the natural LAB population and decreasing the pH of brines until the end of fermentation, and the growth of Enterobacteriaceae and yeast was inhibited. During the storage of fermented green table olives in brine with a potential probiotic strain of *L. pentosus* B281, the growth of the inoculated pathogenic strains such as *E. coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* was suppressed, but they may survive for a long period in such a stressful environment in the fermented product with low pH value (4.2) and high salt concentration (6.0 %) (Argyri et al. 2013). Therefore, application of strict manufacturing practices to reduce the possibility of cross contamination during packaging or post processing of table olives is of significance to ensure the safety of the product.

5.2.3.2 Probiotic Cereal and Soybean Products

Cereals have been considered as a good substrate for the growth of probiotic bacteria, which have been applied in fermentation of different types of traditional cereal products (Table 5.8). Some components of cereals such as water soluble fibers (β-glucan, arabinoxylan, etc.), oligosaccharides (galacto- and fructooligosaccharides, etc.), and resistant starch, may act as prebiotics to selectively stimulate the growth of probiotic microorganisms in the colon (Shah 2001). The viability of probiotic microorganisms may be enhanced in cereal matrices due to the presence of native prebiotics that may protect the probiotics against stress in the gut (Patel et al. 2004). Cereal extracts have also been found to enhance acid and bile tolerance of probiotics (Kalui et al. 2010). Zubaidah et al. (2012) compared the synbiotic effect of fermented rice bran by the probiotic *L. plantarum* B2 and the commercial probiotic *L. casei* in Wistar rats, and found that the fermented rice bran by *L. plantarum* B2 had the prominent synbiotic effect, and production of SCFAs that may decrease the intestine pH to inhibit pathogenic bacteria (*E. coli* and *salmonella*) and prevent the colon cancer.

The functional and sensory properties of cereal-based probiotic products may be dependent on the cereal substrate and the inocula composition. The probiotic strains of *L. acidophilus* and *L. reuteri* were shown to grow better in a malt medium than barley and wheat media due to difference in the chemical composition, though barley and wheat extracts exhibited a significant protective effect on the viability of the probiotics under acidic conditions (Charalampopoulos et al. 2003). A probiotic strain of *L. plantarum* was shown to grow well in the oat bran, a by-product usually discarded by the flour milling industry, but the whole oat flour and white oat flour were more suitable for the probiotic growth (Kedia et al. 2008). *L. plantarum* was also found to grow better in germinated rough rice powder than

Table 5.8 Potential application of probiotics in cereal products

Product	Probiotics	Findings	Reference
Fermented soy product	*L. helveticus, B. longum*	Downregulation of the pro-inflammatory and regulatory cytokines	Masotti et al. (2011)
Fermented soymilk	*L. casei* Zhang	Improved viability of the probiotics in the fermented soymilk with counts higher than 8 Log CFU/ml at 4 °C for 28 days	Wang et al. (2009)
Soy protein bars	*L. acidophilus*	Good survival with probiotic counts of higher than 8 Log CFU/ml at 4 °C for 14 weeks	Chen and Mustapha (2012)
Fermented soy product	*L. acidophilus, B. animalis* subsp. *lactis* Bb-12	Good survival with probiotic counts from 8 to 9 Log CFU/g at 4 °C for 28 days	Bedani et al. (2013)
Fermented soy whey	*L. acidophilus*	The probiotic-fermented soy whey exhibited growth-associated proteolysis and ACE-inhibitory activity	Fung and Liong (2010)
Probiotic-immobilized wheat grains	*L. casei*	Immobilization of the probiotic microorganisms on wheat grains resulted in high cell survival and metabolic activity	Bosnea et al. (2009)
Fermented rice bran	*L. casei, L. plantarum*	High level of fecal viable counts of the probiotics from the rats administered with fermented rice bran	Zubaidah et al. (2012)
Fermented cereal beverages	*L. acidophilus, L. plantarum*	Enhanced growth of the probiotics in malt-containing media; acidification rate varied with the cereal substrates such as malt, barley, and their mixture	Rathore et al. (2012)
Fermented oat beverages	*L. plantarum*	Whole oat flour, white flour, and bran were suitable substrates for probiotic growth with the highest count in white flour (>9 Log CFU/mL) and lowest in bran (>8 Log CFU/mL)	Kedia et al. (2008)
Whole-grain oat drink	*L. plantarum*	High viable cell counts of the probiotic microorganism (>10 Log CFU/mL) with estimated shelf-life of the fermented product under refrigerated storage	Angelov et al. (2006)

the regular rice powder; germination of rice grains was shown to increase the formation of reducing sugars and B vitamins that were needed for the probiotic growth. Rathore et al. (2012) compared the effect of malt and barley flour suspensions, and their mixture on the fermentation of two probiotic strains of *L. plantarum* NCIMB 8826 and *L. acidophilus* NCIMB 8821 at 30 °C for 28 h. The result showed that significant differences in the production of lactic acid were observed between the single and the mixed cereal flours, though similar cell populations were obtained in all these samples. Probiotic fermented maize products may be well accepted by consumers, since maize fermentation was found to induce fruity flavors in traditional Mexican foods (Rivera-Espinoza and Gallardo-Navarro 2010).

The techniques of immobilization of bacterial cells in a suitable carrier have been reported to improve the probiotic viability and stability during processing and storage, and in the gastrointestinal tract (Champagne et al. 2005). The use of cereals or cereal constituents as fermentable substrates may help the growth of probiotic microorganisms due to the presence of specific nondigestible carbohydrates that may function as prebiotics; cereals may also be used as encapsulation materials for protection of probiotics to enhance their stability (Wang et al. 1999). Immobilization of a probiotic microorganism of *L. casei* on boiled wheat grains that was then freeze-dried resulted in high cell survival and metabolic activity during storage at room and low temperatures (4–18 °C) for 9 months. Probiotic microorganisms can also be incorporated into a dry food matrix for long-term storage. Vesterlund et al. (2012) studied the viability of *L. rhamnosus* GG in a crushed flax seed matrix as affected by water activity (a_w) during storage for up to 14 months at room temperature, and found that the viability of the probiotic strain during the storage decreased rapidly at a_w 0.43, and decreased by 2.4 Log_{10} units and 0.29 Log_{10} units at a_w 0.22 and a_w 0.11, respectively. Therefore, storage of probiotics at low water activity may be beneficial to extend the shelf-life of dry probiotic products.

Soy products contain a high content of high-quality protein and polyunsaturated fats, as well as dietary fiber, oligosaccharides, trace mineral and vitamins. Consumption of these products has been considered to be beneficial to reduce the risk of cardiovascular diseases and carcinogenesis, and in the prevention of chronic diseases such as menopausal disorder, atherosclerosis, and osteoporosis (Bedani et al. 2013). Soy products also contain phytoestrogens and isoflavones that may possess bone-sparing effects and anticarcinogenic activity (Donkor et al. 2005). However, there are defects with some soy products, e.g. the presence of unpleasant bean flavor, and the oligosaccharide components such as stachyose and raffinose, which are indigestible and can cause undesirable intestinal symptoms, such as bloating, cramping, and flatulence. Fermentation of soymilk to produce a fermented soy product may improve its texture and flavor, as well as enhance its beneficial health properties (Donkor et al. 2005). Soymilk has been shown to be a good substrate for the growth of many *Lactobacillus* species such *L. casei*, *L. helveticus*, *L. fermenti*, *L. fermentum*, *L. reuteri*, and *L. acidophilus* (Rivera-Espinoza and Gallardo-Navarro 2010). Soymilk may also be a good medium for

the growth of bifidobacteria that are capable of fermenting raffinose and stachyose in the soy products.

Both soymilk and bovine milk were shown to be a good vehicle for delivery of probiotic *L. casei* Zhang. When this probiotic strain was incorporated in the fermented soymilk and bovine milk, it showed good viability (>10^8 CFU/g) during storage at 4 °C for 28d, and tolerated well the stress conditions in simulated gastric juice and intestinal juice. During fermentation of soymilk with *L. casei* Zhang, the pH decreased more rapidly with faster growth of the probiotics when compared with bovine milk at different inoculation rates, probably due to low pH buffering capacity of soymilk (Wang et al. 2009). Farnworth et al. (2007) also reported faster declining of pH in the soy beverage than in the cows' milk when they were fermented with mixed cultures of probiotic *L. rhamnosus* and *L. johnsonii* and the yoghurt strains. The presence of the probiotic bacteria did not influence the growth pattern of the yoghurt strains, which showed synbiotic elements of relationship in soy beverage as in cows' milk. The matrix of fermented soymilk was shown to protect the probiotic *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* La-5 from in vitro simulated gastrointestinal conditions and 28 days of refrigerated storage, with viable counts ranging from 10^8 to 10^9 CFU/g for both microorganisms, but addition of inulin and/or okara flour did not provide further protection for the probiotic microorganisms (Bedani et al. 2013). The presence of oligosaccharides such as α-galactosides (stachyose and raffinose) in soy protein products may be unfavorable for digestion when consumed. Microencapsulation of an α-galactosidase producing *L. acidophilus* LA-2 and subsequently freeze-drying resulted in high viability of the probiotics. Upon incorporation into soy protein bars, the probiotics remained in high numbers throughout 14 weeks of storage at 4 °C, suggesting feasibility of introducing α-galactosidase producing probiotics in a soy food for addressing the problems associated with soy oligosaccharides (Chen and Mustapha 2012).

Soy whey has been evaluated to be a potential alternative to soy milk as a substrate for production of starters for the fermentation of soy products. As shown by Lê et al. (2003), fermentation of soy whey with a *L. paracasei* strain resulted in relatively high viable counts, but most sugars in the whey were not used by this microorganism, and addition of glucose substantially increased the viable counts. The probiotic strain *L. plantarum* 299 V was shown with capability of producing α-galactosidase that catalyzed α-galactosides during fermentation of soymilk (Connes et al. 2004). Ounis et al. (2008) studied the growth of *L. plantarum* LB17 in a partially demineralized pretreated soy whey, and found that the probiotic strain could metabolize more than 60 % of the amounts of stachyose, raffinose, and sucrose contained in the soy whey. The growth of an angiotensin-I converting enzyme (ACE)-producing probiotic strain *L. acidophilus* FTCC 0291 in soy whey exhibited growth-associated proteolysis and ACE-inhibitory activity (Fung and Liong 2010). Soy milk fermented with probiotics, *L. casei, L. acidophilus, L. bulgaricus, S. thermophilus*, and *B. longum* also suggested similarly promising antihypertensive property (Tsai et al. 2006). Soy milk and dairy milk fermented with *S. thermophilus* ST5 in combination with *B. longum* R0175 was shown to

downregulate IL-8 production by HT-29 intestinal epithelial cells, modulate the immune system, prevent infection, reduce symptoms of stress and produce bioactive compounds (Masotti et al. 2011).

5.2.3.3 Probiotic Meat Products

Meat has been considered as a good substrate for probiotic growth, and may provide protection for the probiotics during passage through the gastric intestinal tract (Klingberg and Budde 2006). Dry-fermented meat products are usually not or mildly heated, which may provide suitable conditions for the survival of probiotics, and may act as good carriers for probiotics (Ammor and Mayo 2007). However, the stressful conditions present in the fermented dry meat matrix such as high concentration of salt, acidic pH, and lower water activity may negatively affect the survival of probiotics. Therefore, selection of appropriate probiotic strains for application in meat products is essential to ensure required viability of the probiotics in the final product. Table 5.9 presents some of the recent applications of probiotics in fermented meat products.

Microorganisms naturally found in meat products are considered to be more competitive than other sources for screening probiotic strains due to their better adaption to the meat environment. Ruiz-Moyano et al. (2008) screened among a total of 1000 strains isolated from Iberian dry-fermented sausage (363), pig feces (300), and human feces (337), and found that *L. casei, L. fermentum, L.reuteri, B. animalis, L. murinus, L. vaginalis* and *P. acidilactici* strains exhibited potential probiotic properties, and these strains are capable of growing adequately at the pH values and NaCl concentrations of the fermented sausage product during the ripening process. Pennacchia et al. (2006) evaluated 25 *Lactobacillus* strains isolated from fermented sausage, and found that 8 *L. plantarum* strains and a *L. paracasei* strain possessed potential probiotic properties.

Probiotic cultures can be applied as a substitute to traditional starter cultures or as an adjunst culture in fermented meat products to obtain safe functional food products with probiotic potential. Although the use of probiotic may enhance the functionality of meat products, it should not have negative impacts on the sensory properties of the target products. When the probiotic *B. animalis* 241a and *L. acidophilus* CCDM 476 were used in fermented mutton sausages, the texture and sensory quality of the probiotic products were improved with reduced smell of mutton, and there was a significant increase in viability of the lactobacilli in stool samples after consumption of the probiotic sausages (Holko et al. 2013). The probiotic *B. longum* RO175 and *L. helveticus* RO52 survived well in the fermented sausage with viable counts more than 10^8 CFU/g after ripening for 40 days without significant effect on the chemical composition and sensory quality of the fermented sausages (Radulović et al. 2011). The competitiveness of the probiotic *L. rhamnosus* GG and *L. plantarum* 299 V during the manufacture of Spanish fermented sausages were evaluated by Rubio et al. (2013). The results showed that both strains could prevent the growth of Enterobacteriaceae; *L. plantarum* 299 V

Table 5.9 Potential application of probiotics in fermented meat products

Product	Probiotics	Findings	Reference
Fermented sausages	*L. plantarum, L. rhamnosus*	Good survival with probiotic counts of about 8 Log CFU/g; prevention of the growth of *Enterobacteriaceae* during ripening; no negative effect on physicochemical parameters or sensory attributes of the product	Rubio et al. (2013)
Fermented sausages	*L. helveticus, B. longum*	Increased probiotic growth to around 8 Log CFU/g till the end of ripening; no significant effect on the chemical composition, pH, and sensory quality of the fermented sausages	Radulović et al. (2011)
Fermented mutton sausages	*L. acidophilus, B. animalis*	Better growth of lactobacilli (7 Log CFU/g) than bifidobacteria (3 Log CFU/g); better texture and a reduction of the typical smell of mutton; increased numbers of lactobacilli in stool samples of volunteers	Holko et al. (2013)
Dry-cured pork loins	*L. casei*	Progressive reduction in water activity during aging; no detrimental effects on proteolytic changes; biogenic amines contents varied, but blowed the suggested toxic levels	Stadnik and Dolatowski (2012, 2013)
Dry-fermented pork loins	*L. acidophilus, L. casei*	Good probiotic viability (about 7 Log CFU/g) and satisfying sensory quality of the products after 6 months of storage	Jaworska et al. (2011)
Dry-fermented sausages	*L. fermentum, P. acidilactici*	Good survival of the probiotics during processing; *P.acidilactici* had no notable effects on the physicochemical parameters or sensory quality of the product; acetic acid production and lipid degradation by *L. fermentum* negatively affected the sensory parameters related to color and taste	Ruiz-Moyano et al. (2011)
Scandinavian- type fermented sausages	*L. plantarum, L. pentosus*	High level of probiotic viability (>8 Log CFU/g) after storage at 5 °C for 50 days; no negative effects on flavor; increased capability of passing through GI tract when the probiotics were delivered in the probiotic sausage	Klingberg and Budde (2006), Klingberg et al. (2005)

inoculated at 10^5 CFU/g achieved high counts (ca. 10^8 CFU/g). However, at a higher level of inoculum (ca. 10^7 CFU/g) for both probiotic strains, a decrease of pH values in the fermented sausage was observed, leading to a negative impact on the sensory properties of the product. Likewise, the use of the probiotic *L. fermentum* HL57 in Iberian dry-fermented sausages resulted in increased production of acetic acid and degradation of lipids to form malonaldehyde, and the probiotic product had decreased sensory quality in terms of the color and taste of the product. However, the use of the probiotic *P. acidilactici* SP979 did not affect significantly the physicochemical and sensory properties of Iberian dry-fermented sausages (Ruiz-Moyano et al. 2011).

During manufacturing of fermented meat products, the protein breakdown and lipid degradation in the meat matrix have significant effects on sensory characteristics of the final product (Aro Aro et al. 2010). The free amino acids formed due to proteolysis such as branched-chain amino acids (valine, leucine, and isoleucine), aromatic amino acids (tyrosine, tryptophan, and phenylalanine), and sulfur containing amino acids (methionine and cysteine) are the main aroma compounds in fermented foods (Ardö 2006). Some of the amino acids such as isoleucine, leucine, and valine may be further transformed into methyl-branched aldehydes, alcohols and acids that are strongly linked to dry sausage odor (Olivares and Solano 2009). The endogenous enzymes and/or exogenous enzymes originating from microorganisms may play significant roles in proteolysis of fermented meat products (Aro Aro et al. 2010). Stadnik and Dolatowski (2013) studied the effect of the probiotic *L. casei* ŁOCK 0900 on proteolysis of dry-cured pork loins during aging, and found that the probiotic strain did not have detrimental effects on proteolytic changes in the product. Freiding et al. (2012) investigated the contribution of branched chain aminotransferases to aroma formation from branched chain amino acids, and found that heterologous expression in *L. sakei* TMW1.1322 of the aminotransferase gene significantly increased the conversion of branched chain amino acids to the corresponding alpha-keto-acids. However, the enhanced transaminase activities did not result in increased formation of the methyl-branched volatiles by the *L. sakei* strains, suggesting other factors in addition to the aminotransferase gene that affected aroma formation from amino acids.

The safety of meat and meat products is of great concerns among consumers. Formation of potentially toxic biogenic amines due to the use of poor quality raw materials, unsuitable conditions, and microbial contamination during processing and storage of fermented meat products. Biogenic amines are formed through the decarboxylation of free amino acids or by amination and transamination of aldehydes and ketones (Jansen et al. 2003). The use of probiotic bacteria to suppress the growth of wild amine producing microflora in meat products has been suggested. Stadnik and Dolatowski (2012) investigated the formation of biogenic amines in dry-cured pork loins inoculated with the probiotic *L. casei* ŁOCK 0900, and found that cadaverine (39.6 mg/kg) and tryptamine (49.2 mg/kg) were the main biogenic amines formed, which were below the suggested toxic levels. Recently, *Campylobacter jejuni* from poultry and poultry products has been found

to cause gastroenteritis in humans. Administration of chickens with a *Bifidobacterium*-based synbiotic product containing the probiotic *B. longum* subsp. *longum* PCB133 was shown to increase the beneficial bacteria (i.e., bifidobacteria, lactobacilli) and a significant reduction of *C. jejuni* in the fecal samples. The pathogenic *L. monocytogenes* may cause meat and meat products-related food poisoning. Ingestion of meat starter cultures containing *L. acidophilus* was shown to effectively reduce in the fecal levels of the pathogen (Mahoney and Henriksson 2003).

5.3 Summary and Perspectives

Food fermentation had been practised long before LAB were discovered. Roman historian has described that fermented milk products had been used in the treatment of gastrointestinal infections ever since 76 B.C. In 1907, the concept of probiotics was first proposed by Russian scientist Elie Metchnikoff, and it was hypothesized that consumption of fermented milk would suppress the growth of proteolytic bacteria and reduce putrefaction in the gut, thus prolonging the life span of the host. Later, several food products, supplements, and over-the-counter drugs containing strains of LAB and bifidobacteria were developed for treatment of diarrhea and in the prevention of diseases. The term “probiotic,” which is derived from the Greek/Latin word “pro” and the Greek word “bios,” was first used by Lilly and Stillwell (1965) to depict substances secreted by one organism stimulating the growth of another. According to the recent guidelines of WHO and FAO, probiotics can be defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host.” Probiotic strains are most often from different species of *Lactobacillus* or *Bifidobacterium.*

Probiotic organisms applied in foods are required to be able to survive the gut during passage, being able to resist gastric juices and treatment of bile, to proliferate and colonize the digestive tract. Also, probiotic organisms must be safe and effective, and maintain their effectiveness and potency during the shelf-life of the product. In addition, probiotic strains are preferably of human origin with nonpathogenicity and nontransmissible antibiotic resistance genes, with immunostimulation effects and antagonistic activity against pathogens, but without negative impact on sensory properties of the products. A good probiotic must fulfill the following requirements: (1) ability to adhere to cells; (2) deterring pathogenic adherence; (3) ability to persist, multiply, and produce acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth; (4) being safe, noninvasive, noncarcinogenic, and nonpathogenic; (5) ability to coaggregate as to form a normal-balanced flora.

Lactic acid bacteria have been considered to play important roles in maintaining microbiota balance in the gut. Ingestion of probiotics may exhibit beneficial health effects by modifying the composition of the gut microbiota favoring the species of lactobacilli and bifidobacteria. Efforts are being made with intensive researches on the composition and function of a balanced microbiota in the gut and

their long-term effects on human health. The experimental techniques involve high-throughput microbiota diversity diagnostic arrays, metagenomics, proteomics, metabonomics, and high-throughput phenotyping of metagenomic clones.

Some of the beneficial health effects of probiotics are established from the results of clinical studies, while others are on the basis of in vitro tests. Early clinical studies have been reported with the use of probiotics in the treatment of infectious diseases. During the last decade, probiotics have increasingly been used in therapeutic applications including prevention of urogenital diseases, relieving constipation, protection against traveler's diarrhea and infantile diarrhea, reduction of antibody-induced diarrhea, control of inflammatory bowel diseases and irritable bowel syndrome, reduction of hypercholesterolemia, protection against colon and bladder cancer, prevention of osteoporosis, and prevention of food allergy and atopic diseases.

The efficacy of probiotics is closely related to their interactions with the specific microbiota of the host and with the immuno-competent cells of the intestinal mucosa. The attachment of probiotic microorganisms to the recognition receptors on the surface of the intestinal epithelial cells may cause a cascade of immunological defence mechanisms. Intestinal epithelial cells are believed to be involved in the regulation of the mucosal immune response to bacteria, including pathogens. Ingestion of probiotics may result in the enhancement of innate immune functions including phagocytic activity of neutrophils and cytotoxic activity of NK cells, which might be related to the anti-infectious or anticancer properties of probiotics. Many probiotics may also stimulate the IgAs secreted by B cells and production of cytokines, which are related to communication between macrophages, lymphocytes, and other cells related to the inflammatory reactions and immune responses.

Some probiotics are used for treating inflammatory bowel disease. Probiotic therapy also improves symptoms of irritable bowel syndrome. Several mechanisms may be involved in causing irritable bowel syndrome, including psychosocial factors, altered bowel motility, delicate function of the intestine, imbalance in neurotransmitters, and infection. Other gastrointestinal diseases including diarrhea and food allergies can be effectively treated or prevented by oral probiotic administration. Furthermore, it has been demonstrated that the beneficial effects of probiotics beyond the gut, e.g., positive impact on vaginal health, healing of skin immune homeostasis, improvement of oral malodor parameters; modulation of mood, and stress-induced gastrointestinal symptoms.

Probiotic microorganisms can be incorporated into different types of foods or used in the production of nutraceutical supplements in the form of capsules, tablets, or sachets. Generally, a minimal daily ingestion of viable cells of 10^8–10^9 CFU in the probiotic product is required to exert beneficial health effects in the host. Food substrate may help to buffer the probiotic microorganisms through the stomach, and regulate colonization of probiotics in gastrointestinal tract. The concentration and types of different food components such as fat, protein and sugar, and pH of the product may also influence the growth and survival of the probiotics in foods. Foods may also contain other functional ingredients, e.g., prebiotics, which may interact with probiotics to have their functionality modified.

For effective delivery of probiotic microorganisms to consumers, they must be able to survive food manufacturing conditions, and retain their functionality during food storage. In addition, incorporation of probiotics into foods should not produce off-flavors or have negative effects on textures. The packaging materials and the storage conditions for probiotic products may also be important factors influencing the quality of products, since probiotics, especially bifidobacteria, are often sensitive to oxygen.

Dairy products have been widely used as probiotic carrier foods. Traditionally, probiotic microorganisms are added in yoghurt and other fermented dairy products. In some cases, probiotic culture alone can be used for milk fermentation. Many factors that affect the probiotic viability in yoghurt include the specific probiotic strains used, levels of inoculation, accumulation of acids, interactions between the probiotics and the starter cultures, concentrations of dissolved oxygen and hydrogen peroxide, nutritional components of yoghurt, and storage conditions. The probiotic viability in fermented milk can be enhanced by supplementation with prebiotics and different nutrients, immobilization of the probiotic cells, and use of gases to eliminate oxygen toxicity. Probiotic microorganisms have also been incorporated into traditional fermented milks such as Nordic-cultured buttermilk, Kefir, Viili and Greek-style yoghurt, etc.

Other dairy products proved to be suitable carriers of probiotics include cheeses, dairy beverage, ice cream, etc. The cheese matrix creates better environment than fermented milk for probiotic survival. However, there are different compositional and processing factors that may significantly influence the probiotic viability in cheese, including strains of probiotic cultures, rate of inoculation, supplementation of milk with nutrients, incubation temperature, pH, titratable acidity, hydrogen peroxide, bacteriocins, flavoring agents, microbial competitions, metabolite concentrations like lactic and acetic acids, dissolved oxygen, storage temperature, addition of salt and antimicrobial preservatives, and packaging materials. The probiotic viability in dairy beverage relies on the specific strain used, and its interaction with other strains of the starter cultures, as well as variation of the food matrix of the product. Production of ice cream involves freezing and thawing operations that may damage cells, and also the stresses of oxygen mixed and incorporated into the mixture may cause decreased functionality of the product. Selection of stable probiotic strains is of significance to assure enough viability of the probiotics in the frozen products. One way to improve the probiotic survival in the hostile environment and gastrointestinal tract is by physical protection of probiotics via microencapsulation of the bacterial cells.

The increasing consumer health demand has stimulated exploring various nondairy products, including fruits, vegetables, cereals, legumes, and meat. These products may provide alternative forms of probiotics for vegetarians, consumers with lactose intolerance or dyslipidemia, and those who prefer low cholesterol diets.

Fruits and vegetables are rich in nutrients with taste profiles appealing to all individuals. By modification of their food components and structures, fruits and

vegetables can serve as ideal carriers for probiotics. In addition, these foods generally contain high amount of dietary fibers. Some of the dietary fibers may function as prebiotics that may promote the survival of probiotic bacteria during passing through the gastrointestinal tract. Vacuum impregnation can be used to introduce probiotics into the porous structure of fruits and vegetables. The gas and liquid filled in the intercellular spaces can be removed by vacuum and replaced by diffusion with the probiotics. This technique has been successfully used in apples. Regarding applications in fruit and vegetable juices, the viability of probiotics may be negatively influenced by the low pH and levels of organic acids, and the presence of total phenol and oxygen.

Cereals have been evaluated to be suitable for growth of probiotic LAB. Functional food formulations often include cereals as substrates for probiotic growth, as prebiotics due to content of nondigestible carbohydrates, and also as materials for encapsulation of probiotics to enhance their stability. The presence of native prebiotics in cereal matrices may protect the probiotics against stress in the gut. Factors to be considered for developing probiotic cereal products are the composition of cereal grains, the substrate formulation, selection of probiotic strains, the nutritional and sensory properties of the final product. Soymilk has been shown to be good for the growth of many *Lactobacillus* species such *L. casei, L. helveticus, L. fermenti, L. fermentum, L. reuteri*, and *L. acidophilus*. Soymilk may also be a good medium for the growth of bifidobacteria that are capable of fermenting raffinose and stachyose in the soy products.

Dry-fermented meat products are often not heated, creating suitable conditions for probiotic survival. However, the stressful conditions present in the fermented dry meat matrix such as high concentration of salt, acidic pH, and lower water activity may negatively affect the survival of probiotics. The safety of meat and meat products is of great concern among consumers. There has been an increasing challenge in meat industry regarding the illegal use of poor quality raw materials that may increase the potential to form toxic biogenic amines during processing and storage of fermented meat products. It has been suggested that the use of probiotic bacteria may provide effective ways to suppress the growth of microflora that produces wild amines in meat products.

Nowadays, LAB as the major source of probiotics have been gaining significance due to numerous beneficial health effects resulted from consumption of probiotic foods or nutraceutical supplements. Particularly, the health benefits of probiotics related to effective immune system and gut comfort in the general healthy adult population has increased dramatically the market potential during recent years. The success of probiotic marketing can be attributed to the continuous accumulation of scientific evidence, the increased consumer understanding of these health benefits, and demands for the probiotic products. However, the presence of probiotic strains in different fermented products should be better identified and characterized using more sophisticated biomolecular approaches. High-quality data are needed to confirm probiotic efficacy related to specific health claims of probiotic products. With more information of whole genome sequence of

probiotic strains, better understanding of the mechanism of probiotic action and the roles of probiotics in promoting various health benefits can be achieved.

Traditionally, probiotic candidates are selected on the basis of a procedure involving a series of in vitro assays and preclinical tests that may include in vitro or in vivo modulation of immune cell function and gastrointestinal microbiota. However, more systematic investigation and characterization with better predictive in vitro assays and well-designed human trials are needed in order to develop successful probiotics with demonstrated health benefits. The screening of strains with better probiotic phenotype could be performed from more wide sources, though many of the currently isolated probiotic strains are of human origin. The present starter strains from dairy and other fermentations can also be screened to obtain unique properties of a fermented food with desired taste, flavor, safety, color, texture, nutritional value, and health benefits.

Probiotic strains could be improved via different strategies, e.g., by taking advantage of bacterial surface properties, or manipulating metabolic pathways. The cell wall carbohydrates and exopolysaccharides have been identified as adhesion factors, which may affect bacterial colonization in the intestine. Extracellular macromolecules also offer protection for the bacterial strains against harsh environmental conditions. Therefore, factors favoring maintenance of surface properties during manufacturing and gastrointestinal transit may be beneficial for the enhancement of probiotic efficacy. The probiotic properties can also be altered by manipulation of metabolic pathways in the bacteria. For instance, the bile tolerance of probiotic strain could be enhanced by induced expression of bile salt hydrolase. Aerotolerance of bifidobacteria can be enhanced by induction of NADH oxidases and peroxidases that may help remove reactive oxygen species. Process of tolerance adaption, e.g. to low pH or bile salts, often is accompanied with increased synthesis of chaperones, and detection of increased level of chaperone in the cytoplasm indicates better tolerance to stress conditions by the particular probiotic strain.

Monitoring the stability of probiotics during manufacturing process and storage is of vital importance in order to produce probiotics with required functional properties. Traditional plate counting methodology often underestimates the cell numbers of the probiotic products because these cells are typically stressed during processing and storage. Some treatments, e.g. microencapsulation of cells, may require modifications of sampling procedure to release the cells. To estimate viable cells precisely, specific analytic procedures should be followed. The recommended viability determination of probiotics in the commercial product such as powders, frozen concentrates, microencapsulated cultures, capsules, foods and beverages have recently been reviewed by Champagne et al. (2011).

The safety of potential probiotics should be assessed before they can be subjected to applications in foods. Several guidelines can be followed for the safety assessment of microorganisms, e.g. the European system of Qualified Presumption of Safety, and the US system of Generally Recognized As Safe. The required data for safety assessment may vary with the bacterial species, the expected application and the target populations. Generally, factors to be considered in the safety

evaluation of probiotics include identity of the strain, occurrence of disease, history of use in food, toxic or metabolic effects on the gastrointestinal tract, transfer of antibiotic resistance genes in the flora in gastrointestinal tract. Many *Lactobacillus* strains with intrinsic vancomycin resistance have a long history of safe use, and no transfer of the resistance to other bacteria has been reported. However, there are sometimes virulence factors present in probiotics, acquisition in probiotic bacteria of virulence genes or antimicrobial resistance due to horizontal gene transfer, and spread of resistance that is unwanted in endogenous bacterial populations. The safety related to possible presence of plasmid-linked antibiotic resistance in some lactobacilli, though not common, should also be taken into consideration. Furthermore, antibiotic susceptibility patterns vary widely with different species and strains of lactobacilli, and they should be evaluated for each strain of interest.

The composition of human gut microbiota is closely related to human diet and the physiological conditions of the host. Diet is not only the nutrient source for the host, but also for the gastrointestinal microflora, which metabolize the dietary components undigested by the host. Currently most work on characterization of the intestinal microbial composition is limited to individuals under a western diet. It is important to study the intestinal microbes from populations in different regions of the world to better define the human gut microbiota. For instance, more Bacteroidetes but less Firmicutes have been found in the gut microbes of African people probably due to their fiber-rich diet. In addition, the gut microbiota can be affected by different disturbance events, e.g., antibiotic therapy, drug treatments, surgery, and disease. It is therefore necessary to assess the variation of the gut microbial community under a wide range of physiological conditions.

It has been reported that gut microbiota plays significant roles in the intestinal development, homeostasis, energy harvesting by the host, and protection against pathogens (Montalto et al. 2009). However, obesity in rodents and humans has been reported to associate with a 50 % reduction of Bacteroidetes and an increase in Firmicutes, suggesting changes in population of gut microbiota could lead to differences in metabolism of substrates and energy harvest (Ley et al. 2005, 2006). Metabolic activities of gut microbiota may also result in the production of harmful substances, e.g., ammonia, hydrogen sulfide, nitrosamines, phenols, indoles, and deoxycholic acid, which are involved in carcinogenesis. Therefore, further characterization of gut microflora composition, and the dynamic interactions between the microbiota and host tissue will be crucial for understanding both the beneficial and harmful roles of gut microbiota in human health and diseases.

Oral administration of probiotics plays beneficial roles in the modulation of gut microflora, which may lead to more "good" microbes than "bad" microbes in the gut. Although almost all currently used probiotics do not permanently reside in the gut, their temporary presence during digest in gut can have modulating effects, which may benefit human health. For instance, the probiotic *L. helveticus* and *L. acidophilus* have been shown to reduce the ability of *E. coli* O157:H7 to attach to epithelial cells, and thus prevent the pathogenesis of disease caused by this pathogen. The probiotic *L. casei* DN-114001 is capable of eradicating the presence

of *H. pylori*, which is associated with the manifestation of gastric adenocarcinoma. Probiotics have also been shown to inhibit the growth and colonization of pathogens in the gut, and have been used to treat intestinal ailments such as food-borne pathogens, antibiotic-associated diarrhea, and *C. difficile*-associated diarrhea. However, there are challenges with the applications of probiotics in health and clinical practice. For example, the beneficial effects of currently used probiotics are generally case-specific, and no single probiotic has been identified with effectiveness for all individuals. The mechanism of specific probiotic-related action and the sustainability of the effectiveness are not clearly known. Furthermore, previous trials with probiotics are often of small size with limitations of methodology, and many studies have not been reproduced.

With rapid development of "omics" technologies and availability of full genome sequences of several probiotic species, the molecular mechanisms underlining the probiotic health effects will be further elucidated. Studies of proteomics may provide information regarding protein biological functions, stress response and adhesion mechanisms. Novel approaches of metabolomics can be employed to study several effector molecules associated with probiotic cell envelope that are involved in immunomodulation and cell signaling. The advent of new technologies with high-throughput pyrosequencing and metagenomics may facilitate identification of all microbial community members and their mutual interactions in the gut. Future clinical trials with probiotics at a large scale combining with more sophisticated methodology as mentioned above will help reach definite evidence of the preventive and curative roles of probiotics related to human health and disease.

References

Abrahamsson TR, Jakobsson T, Böttcher MF, Fredrikson M, Jenmalm MC, Björkstén B, Oldaeus G. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol. 2007;119:1174–118090.

Abrahamsson TR, Jakobsson T, Björkstén B, Oldaeus G, Jenmalm MC. No effect of probiotics on respiratory allergies: a seven-year follow-up of a randomized controlled trial in infancy. Pediatr Allergy Immunol. 2013;24:556–61.

Adams CA. The probiotic paradox: live and dead cells are biological response modifiers. Nutr Res Rev. 2010;23:37–46.

Aggarwal J, Swami G, Kumar M. Probiotics and their effects on metabolic diseases: an update. J Clin Diagn Res JCDR. 2013;7:173–7.

Aguilera JM. Microstructural principles of food processing and engineering. 2nd ed. Gaithersburg, MD: Aspen Publishers; 1999.

Akalın AS, Tokuşoğlu ö, Gönç S, Aycan ş. Occurrence of conjugated linoleic acid in probiotic yoghurts supplemented with fructooligosaccharide. Int Dairy J. 2007;17:1089–1095.

Akalin AS, Erişir D. Effects of inulin and oligofructose on the rheological characteristics and probiotic culture survival in low-fat probiotic ice cream. J Food Sci. 2008;73:M184–8.

Akalın AS, Unal G, Dinkci N, Hayaloglu AA. Microstructural, textural, and sensory characteristics of probiotic yogurts fortified with sodium calcium caseinate or whey protein concentrate. J Dairy Sci. 2012;95:3617–28.

Akatsu H, Iwabuchi N, Xiao J-Z, Matsuyama Z, Kurihara R, Okuda K, Yamamoto T, Maruyama M. Clinical effects of probiotic *Bifidobacterium longum* BB536 on immune function and intestinal microbiota in elderly patients receiving enteral tube feeding. JPEN J Parenter Enteral Nutr. 2013;37:631–40.

Alamprese C, Foschino R, Rossi M, Pompei C, Savani L. Survival of *Lactobacillus johnsonii* La1 and influence of its addition in retail-manufactured ice cream produced with different sugar and fat concentrations. Int Dairy J. 2002;12:201–8.

Alegre I, Viñas I, Usall J, Anguera M, Abadias M. Microbiological and physicochemical quality of fresh-cut apple enriched with the probiotic strain *Lactobacillus rhamnosus* GG. Food Microbiol. 2011;28:59–66.

Allen SJ, Wareham K, Wang D, et al. Lactobacilli and *Bifidobacteria* in the prevention of antibiotic-associated diarrhoea and *Clostridium difficile* diarrhoea in older inpatients (PLACIDE): a randomised, double-blind, placebo-controlled, multicentre trial. The Lancet. 2013;382:1249–57.

Allgeyer LC, Miller MJ, Lee SY. Sensory and microbiological quality of yogurt drinks with prebiotics and probiotics. J Dairy Sci. 2010;93:4471–9.

Almeida KE, Tamime AY, Oliveira MN. Acidification rates of probiotic bacteria in Minas frescal cheese whey. LWT Food Sci Technol. 2008;41:311–6.

Almeida KE, Tamime AY, Oliveira MN. Influence of total solids contents of milk whey on the acidifying profile and viability of various lactic acid bacteria. LWT Food Sci Technol. 2009;42:672–8.

Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 1995;59:143–69.

Ammor MS, Mayo B. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: an update. Meat Sci. 2007;76:138–46.

Anderson JW, Gilliland SE. Effect of fermented milk (yogurt) containing *Lactobacillus acidophilus* L1 on serum cholesterol in hypercholesterolemic humans. J Am Coll Nutr. 1999;18:43–50.

Angelov A, Gotcheva V, Kuncheva R, Hristozova T. Development of a new oat-based probiotic drink. Int J Food Microbiol. 2006;112:75–80.

Ankolekar C, Pinto M, Greene D, Shetty K. In vitro bioassay based screening of antihyperglycemia and antihypertensive activities of *Lactobacillus acidophilus* fermented pear juice. Innov Food Sci Emerg Technol. 2012;13:221–30.

Araújo EA, de Carvalho AF, Leandro ES, Furtado MM, de Moraes CA. Development of a symbiotic cottage cheese added with *Lactobacillus delbrueckii* UFV H2b20 and inulin. J Funct Foods. 2010;2:85–9.

Ardö Y. Flavour formation by amino acid catabolism. Biotechnol Adv. 2006;24:238–42.

Argyri AA, Lyra E, Panagou EZ, Tassou CC. Fate of *Escherichia coli* O157:H7, *Salmonella Enteritidis* and *Listeria monocytogenes* during storage of fermented green table olives in brine. Food Microbiol. 2013;36:1–6.

Aro Aro JM, Nyam-Osor P, Tsuji K, Shimada K, Fukushima M, Sekikawa M. The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages. Food Chem. 2010;119:279–85.

Asahara T, Shimizu K, Nomoto K, Hamabata T, Ozawa A, Takeda Y. Probiotic *Bifidobacteria* protect mice from lethal infection with Shiga toxin-producing *Escherichia coli* O157:H7. Infect Immun. 2004;72:2240–7.

Asemi Z, Zare Z, Shakeri H, Sabihi S-S, Esmaillzadeh A. Effect of multispecies probiotic supplements on metabolic profiles, hs-CRP, and oxidative stress in patients with type 2 diabetes. Ann Nutr Metab. 2013;63:1–9.

Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci USA. 2004;101:15718–23.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307:1915–20.

Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun. 2011;25:397–407.

Balabanova T, Panayotov P. Obtaining functional fermented beverages by using the kefir grains. Procedia Food Sci. 2011;1:1653–9.

Başyiğit G, Kuleaşan H, Karahan AG. Viability of human-derived probiotic lactobacilli in ice cream produced with sucrose and aspartame. J Ind Microbiol Biotechnol. 2006;33:796–800.

Bedani R, Rossi EA, Isay Saad SM. Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under in vitro simulated gastrointestinal conditions. Food Microbiol. 2013;34:382–389.

Ben Ounis W, Champagne CP, Makhlouf J, Bazinet L. Utilization of tofu whey pre-treated by electromembrane process as a growth medium for *Lactobacillus plantarum* LB17. Desalination. 2008;229:192–203.

Bercik P, Verdu EF, Foster JA, et al. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology. 2010;139(2102–2112):e1.

Bercik P, Park AJ, Sinclair D, et al. The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil. 2011;23:1132–9.

Betoret E, Betoret N, Arilla A, Bennár M, Barrera C, Codoñer P, Fito P. No invasive methodology to produce a probiotic low humid apple snack with potential effect against *Helicobacter pylori*. J Food Eng. 2012;110:289–93.

Blaiotta G, Di Capua M, Coppola R, Aponte M. Production of fermented chestnut purees by lactic acid bacteria. Int J Food Microbiol. 2012;158:195–202.

Bosnea LA, Kourkoutas Y, Albantaki N, Tzia C, Koutinas AA, Kanellaki M. Functionality of freeze-dried *L. casei* cells immobilized on wheat grains. LWT - Food Sci Technol. 2009;42:1696–702.

Bottazzi V, Zacconi C, Gonzaga E, Paladino M. Absorption of cholesterol by intestinal lactic acid bacteria. Ann Microbiol. 1986;36:1–5.

Boyle RJ, Bath-Hextall FJ, Leonardi-Bee J, Murrell DF, Tang ML-K. Probiotics for the treatment of eczema: a systematic review. Clin Exp Allergy. 2009;39:1117–27.

Boyle RJ, Ismail IH, Kivivuori S, et al. *Lactobacillus* GG treatment during pregnancy for the prevention of eczema: a randomized controlled trial. Allergy. 2011;66:509–16.

Boylston TD, Vinderola CG, Ghoddusi HB, Reinheimer JA. Incorporation of *Bifidobacteria* into cheeses: challenges and rewards. Int Dairy J. 2004;14:375–87.

Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci USA. 2011;108:16050–5.

Buriti FCA, Castro IA, Saad SMI. Viability of *Lactobacillus acidophilus* in synbiotic guava mousses and its survival under in vitro simulated gastrointestinal conditions. Int J Food Microbiol. 2010a;137:121–9.

Buriti FCA, Castro IA, Saad SMI. Effects of refrigeration, freezing and replacement of milk fat by inulin and whey protein concentrate on texture profile and sensory acceptance of synbiotic guava mousses. Food Chem. 2010b;123:1190–7.

Camilleri M. Probiotics and irritable bowel syndrome: rationale, putative mechanisms, and evidence of clinical efficacy. J Clin Gastroenterol. 2006;40:264–9.

Cani PD, Delzenne NM. Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharmacol. 2009;9:737–43.

Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM. Selective increases of *Bifidobacteria* in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50:2374–83.

Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008;57:1470–81.

Cani PD, Possemiers S, Van de Wiele T, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58:1091–103.

Cardarelli HR, Buriti FCA, Castro IA, Saad SMI. Inulin and oligofructose improve sensory quality and increase the probiotic viable count in potentially synbiotic petit-suisse cheese. LWT Food Sci Technol. 2008;41:1037–46.

Castro WF, Cruz AG, Bisinotto MS, Guerreiro LMR, Faria JAF, Bolini HMA, Cunha RL, Deliza R. Development of probiotic dairy beverages: rheological properties and application of mathematical models in sensory evaluation. J Dairy Sci. 2013a;96:16–25.

Castro WF, Cruz AG, Rodrigues D, Ghiselli G, Oliveira CAF, Faria JAF, Godoy HT. Short communication: effects of different whey concentrations on physicochemical characteristics and viable counts of starter bacteria in dairy beverage supplemented with probiotics. J Dairy Sci. 2013b;96:96–100.

Champagne CP, Gardner NJ, Roy D. Challenges in the addition of probiotic cultures to foods. Crit Rev Food Sci Nutr. 2005;45:61–84.

Champagne CP, Ross RP, Saarela M, Hansen KF, Charalampopoulos D. Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. Int J Food Microbiol. 2011;149:185–93.

Charalampopoulos D, Pandiella SS, Webb C. Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. Int J Food Microbiol. 2003;82:133–41.

Chen M, Mustapha A. Survival of freeze-dried microcapsules of α-galactosidase producing probiotics in a soy bar matrix. Food Microbiol. 2012;30:68–73.

Chen Y-S, Jan R-L, Lin Y-L, Chen H-H, Wang J-Y. Randomized placebo-controlled trial of *Lactobacillus* on asthmatic children with allergic rhinitis. Pediatr Pulmonol. 2010;45:1111–20.

Cliff MA, Fan L, Sanford K, Stanich K, Doucette C, Raymond N. Descriptive analysis and early-stage consumer acceptance of yogurts fermented with carrot juice. J Dairy Sci. 2013;96:4160–72.

Collins JK, Thornton G, Sullivan GO. Selection of probiotic strains for human applications. Int Dairy J. 1998;8:487–90.

Connes C, Silvestroni A, Leblanc JG, Juillard V, Savoy de Giori G, Sesma F, Piard J-C. Towards probiotic lactic acid bacteria strains to remove raffinose-type sugars present in soy-derived products. Le Lait. 2004;84:207–214.

Conway PL, Gorbach SL, Goldin BR. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J Dairy Sci. 1987;70:1–12.

Costa MGM, Fonteles TV, de Jesus ALT, Rodrigues S. Sonicated pineapple juice as substrate for *L. casei* cultivation for probiotic beverage development: process optimisation and product stability. Food Chem. 2013;139:261–6.

Cruz AG, Alonso Buriti FC, Batista de Souza CH, Fonseca Faria JA, Isay Saad SM. Probiotic cheese: health benefits, technological and stability aspects. Trends Food Sci Technol. 2009a;20:344–54.

Cruz AG, Antunes AEC, Sousa ALOP, Faria JAF, Saad SMI. Ice-cream as a probiotic food carrier. Food Res Int. 2009b;42:1233–9.

Cruz AG, Fonseca Faria J de A, Isay Saad SM, André Bolini HM, Sant′Ana AS, Cristianini M. High pressure processing and pulsed electric fields: potential use in probiotic dairy foods processing. Trends Food Sci Technol. 2010;21:483–493.

Davari S, Talaei SA, Alaei H, Salami M. Probiotics treatment improves diabetes-induced impairment of synaptic activity and cognitive function: behavioral and electrophysiological proofs for microbiome-gut-brain axis. Neuroscience. 2013;240:287–96.

Dave RI, Shah NP. Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. Int Dairy J. 1997;7:31–41.

Dave RI, Shah NP. Ingredient supplementation effects on viability of probiotic bacteria in yogurt. J Dairy Sci. 1998;81:2804–16.

De Azeredo GA, Stamford TLM, Nunes PC, Gomes Neto NJ, de Oliveira MEG, de Souza EL. Combined application of essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. Food Res Int. 2011;44:1541–1548.

De Bellis P, Valerio F, Sisto A, Lonigro SL, Lavermicocca P. Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. Int J Food Microbiol. 2010;140:6–13.

De Castro FP, Cunha TM, Ogliari PJ, Teófilo RF, Ferreira MMC, Prudêncio ES. Influence of different content of cheese whey and oligofructose on the properties of fermented lactic beverages: study using response surface methodology. LWT Food Sci Technol. 2009;42:993–7.

De Greef E, Vandenplas Y, Hauser B, Devreker T, Veereman-Wauters G. Probiotics and IBD. Acta Gastro-Enterol Belg. 2013;76:15–9.

De Vrese M, Winkler P, Rautenberg P, et al. Effect of *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, *B. bifidum* MF 20/5 on common cold episodes: a double blind, randomized, controlled trial. Clin Nutr (Edinburgh, Scotland). 2005;24:481–91.

Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression. Neuroscience. 2010;170:1179–88.

Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci USA. 2011;108:3047–52.

Do Espírito Santo AP, Cartolano NS, Silva TF, Soares FASM, Gioielli LA, Perego P, Converti A, Oliveira MN. Fibers from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yoghurts. Int J Food Microbiol. 2012a;154:135–44.

Do Espírito Santo AP, Perego P, Converti A, Oliveira MN. Influence of milk type and addition of passion fruit peel powder on fermentation kinetics, texture profile and bacterial viability in probiotic yoghurts. LWT Food Sci Technol. 2012b;47:393–9.

Donkor ON, Henriksson A, Vasiljevic T, Shah NP. Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yogurt. J Food Sci. 2005;70:m375–81.

Donkor ON, Henriksson A, Vasiljevic T, Shah NP. Rheological properties and sensory characteristics of set-type soy yogurt. J Agric Food Chem. 2007;55:9868–76.

Dotterud CK, Storrø O, Johnsen R, Oien T. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. Br J Dermatol. 2010;163:616–23.

Ebel B, Martin F, Le LDT, Gervais P, Cachon R. Use of gases to improve survival of *Bifidobacterium bifidum* by modifying redox potential in fermented milk. J Dairy Sci. 2011;94:2185–91.

EFSA (European Food Safety Authority). Opinion of the scientific committee on a request from EFSA on the introduction of a qualified presumption of safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J. 2007;587:1–16.

EFSA (European Food Safety Authority). Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2011 update). EFSA J. 2011;9:2497.

EFSA. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Eur Food Saf Auth. 2012;10:2740.

Ejtahed HS, Mohtadi-Nia J, Homayouni-Rad A, Niafar M, Asghari-Jafarabadi M, Mofid V, Akbarian-Moghari A. Effect of probiotic yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium lactis* on lipid profile in individuals with type 2 diabetes mellitus. J Dairy Sci. 2011;94:3288–94.

Ejtahed HS, Mohtadi-Nia J, Homayouni-Rad A, Niafar M, Asghari-Jafarabadi M, Mofid V. Probiotic yogurt improves antioxidant status in type 2 diabetic patients. Nutrition (Burbank, Los Angeles County, California). 2012;28:539–43.

Elazab N, Mendy A, Gasana J, Vieira ER, Quizon A, Forno E. Probiotic administration in early life, atopy, and asthma: a meta-analysis of clinical trials. Pediatrics. 2013;132:e666–76.

Elizaquível P, Sánchez G, Salvador A, Fiszman S, Dueñas MT, López P, Fernández de Palencia P, Aznar R. Evaluation of yogurt and various beverages as carriers of lactic acid bacteria producing 2-branched (1,3)-β-D-glucan. J Dairy Sci. 2011;94:3271–3278.

Escobar MC, Van Tassell ML, Martínez-Bustos F, Singh M, Castaño-Tostado E, Amaya-Llano SL, Miller MJ. Characterization of a Panela cheese with added probiotics and fava bean starch. J Dairy Sci. 2012;95:2779–87.

Espírito-Santo AP, Lagazzo A, Sousa ALOP, Perego P, Converti A, Oliveira MN. Rheology, spontaneous whey separation, microstructure and sensorial characteristics of probiotic yoghurts enriched with passion fruit fiber. Food Res Int. 2013;50:224–31.

Everard A, Belzer C, Geurts L, et al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci USA. 2013;110:9066–71.

Farnworth ER, editor. Handbook of fermented functional foods. Boca Raton: CRC Press; 2003.

Farnworth ER, Mainville I, Desjardins MP, Gardner N, Fliss I, Champagne C. Growth of probiotic bacteria and *Bifidobacteria* in a soy yogurt formulation. Int J Food Microbiol. 2007;116:174–81.

Fiocchi A, Burks W, Bahna SL, et al. Clinical use of probiotics in pediatric allergy (CUPPA): a world allergy organization position paper. World Allergy Organ J. 2012;5:148–67.

Food and Agriculture Organization of the United Nations and World Health Organization. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid, 2001 bacteria. http://www.fao.org/es/ESN/food/food_probio_en.stm

Food and Agriculture Organization of the United Nations and World Health Organization. Guidelines for the evaluation of probiotics in food. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food, 2002.

Freiding S, Ehrmann MA, Vogel RF. Comparison of different IlvE aminotransferases in *Lactobacillus sakei* and investigation of their contribution to aroma formation from branched chain amino acids. Food Microbiol. 2012;29:205–14.

Fu N, Chen XD. Towards a maximal cell survival in convective thermal drying processes. Food Res Int. 2011;44:1127–49.

Fukuda S, Toh H, Hase K, et al. *Bifidobacteria* can protect from enteropathogenic infection through production of acetate. Nature. 2011;469:543–7.

Fuller R. Probiotics in man and animals. J Appl Bacteriol. 1989;66:365–78.

Fung WY, Liong MT. Evaluation of proteolytic and ACE-inhibitory activity of *Lactobacillus acidophilus* in soy whey growth medium via response surface methodology. LWT Food Sci Technol. 2010;43:563–7.

Gangadharan D, Nampoothiri KM. Folate production using *Lactococcus lactis* ssp *cremoris* with implications for fortification of skim milk and fruit juices. LWT Food Sci Technol. 2011;44:1859–64.

Gauffin Cano P, Santacruz A, Moya Á, Sanz Y. *Bacteroides uniformis* CECT 7771 ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. PLoS ONE. 2012;7:e41079.

Gill HS, Rutherfurd KJ, Cross ML, Gopal PK. Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. Am J Clin Nutr. 2001;74:833–9.

Giovannini M, Agostoni C, Riva E, Salvini F, Ruscitto A, Zuccotti GV, Radaelli G, Felicita Study Group. A randomized prospective double blind controlled trial on effects of long-term consumption of fermented milk containing *Lactobacillus casei* in pre-school children with allergic asthma and/or rhinitis. Pediatr Res. 2007;62:215–220.

Gobbetti M, Corsetti A, Smacchi E, Zocchetti A, De Angelis M. Production of Crescenza Cheese by incorporation of *Bifidobacteria*. J Dairy Sci. 1998;81:37–47.

Grundy SM. Treatment of hypercholesterolemia by interference with bile acid metabolism. Arch Intern Med. 1972;130:638–48.

Guergoletto KB, Magnani M, Martin JS, Andrade CGT de J, Garcia S. Survival of *Lactobacillus casei* (LC-1) adhered to prebiotic vegetal fibers. Innov Food Sci Emerg Technol. 2010;11:415–421.

Guo M (2003) Goat's milk encyclopedia of food sciences and nutrition. Oxford: Elsevier; 2003, pp. 2944–2949.

Guo Z, Liu XM, Zhang QX, Shen Z, Tian FW, Zhang H, Sun ZH, Zhang HP, Chen W. Influence of consumption of probiotics on the plasma lipid profile: a meta-analysis of randomised controlled trials. Nutr Metab Cardiovasc Dis NMCD. 2011;21:844–50.

Gupta P, Andrew H, Kirschner BS, Guandalini S. Is *Lactobacillus* GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. J Pediatr Gastroenterol Nutr. 2000;31:453–7.

Hara H, Haga S, Kasai T, Kiriyama S. Fermentation products of sugar-beet fiber by cecal bacteria lower plasma cholesterol concentration in rats. J Nutr. 1998;128:688–93.

Hazebrouck S, Pothelune L, Azevedo V, Corthier G, Wal J-M, Langella P. Efficient production and secretion of bovine beta-lactoglobulin by *Lactobacillus casei*. Microb Cell Factories. 2007;6:12.

Hedin C, Whelan K, Lindsay JO. Evidence for the use of probiotics and prebiotics in inflammatory bowel disease: a review of clinical trials. Proc Nutr Soc. 2007;66:307–15.

Heidebach T, Först P, Kulozik U. Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. J Food Eng. 2010;98:309–16.

Hekmat S, Soltani H, Reid G. Growth and survival of *Lactobacillus reuteri* RC-14 and *Lactobacillus rhamnosus* GR-1 in yogurt for use as a functional food. Innov Food Sci Emerg Technol. 2009;10:293–6.

Hemsworth J, Hekmat S, Reid G. The development of micronutrient supplemented probiotic yogurt for people living with HIV: laboratory testing and sensory evaluation. Innov Food Sci Emerg Technol. 2011;12:79–84.

Hill MJ. Intestinal flora and endogenous vitamin synthesis. Eur J Cancer Prev. 1997;6(Suppl 1):S43–5.

Hol J, van Leer EHG, Elink Schuurman BEE, de Ruiter LF, Samsom JN, Hop W, Neijens HJ, de Jongste JC, Nieuwenhuis EES, Cow's Milk Allergy Modified by Elimination and Lactobacilli study group. The acquisition of tolerance toward cow's milk through probiotic supplementation: a randomized, controlled trial. J Allergy Clin Immunol. 2008;121:1448–1454.

Holko I, Hrabě J, Šalaková A, Rada V. The substitution of a traditional starter culture in mutton fermented sausages by *Lactobacillus acidophilus* and *Bifidobacterium animalis*. Meat Sci. 2013;94:275–9.

Homayouni A, Azizi A, Ehsani MR, Yarmand MS, Razavi SH. Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. Food Chem. 2008;111:50–5.

Hosono A, Tono-oka T. Binding of cholesterol with lactic acid bacterial cells. Milchwissenschaft. 1995;50:556–60.

Hsieh F-C, Lee C-L, Chai C-Y, Chen W-T, Lu Y-C, Wu C-S. Oral administration of *Lactobacillus reuteri* GMNL-263 improves insulin resistance and ameliorates hepatic steatosis in high fructose-fed rats. Nutr Metab. 2013;10:35.

Huang JS, Bousvaros A, Lee JW, Diaz A, Davidson EJ. Efficacy of probiotic use in acute diarrhea in children: a meta-analysis. Dig Dis Sci. 2002;47:2625–34.

IDF Diabetes Atlas, Fifth Edition.

Isolauri E, Arvola T, Sütas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. Clin Exp Allergy. 2000;30:1604–10.

Jankovic M, Robbiani DF, Dorsett Y, Eisenreich T, Xu Y, Tarakhovsky A, Nussenzweig A, Nussenzweig MC. Role of the translocation partner in protection against AID-dependent chromosomal translocations. Proc Natl Acad Sci USA. 2010;107:187–92.

Jansen SC, van Dusseldorp M, Bottema KC, Dubois AEJ. Intolerance to dietary biogenic amines: a review. Ann Allergy Asthma Immunol.2003;91:233–240; quiz 241–242, 296.

Jaworska D, Neffe K, Kołożyn-Krajewska D, Dolatowski Z. Survival during storage and sensory effect of potential probiotic lactic acid bacteria *Lactobacillus acidophilus* Bauer and *Lactobacillus casei* Bif3′/ IV in dry fermented pork loins: probiotics in the dry fermented loins. Int J Food Sci Technol. 2011;46:2491–7.

Jayamanne VS, Adams MR. Determination of survival, identity and stress resistance of probiotic *Bifidobacteria* in bio-yoghurts. Lett Appl Microbiol. 2006;42:189–94.

Jin J-S, Kitahara M, Sakamoto M, Hattori M, Benno Y. *Slackia equolifaciens* sp. nov., a human intestinal bacterium capable of producing equol. Int J Syst Evol Microbiol. 2010;60:1721–4.

Johnston BC, Ma SSY, Goldenberg JZ, Thorlund K, Vandvik PO, Loeb M, Guyatt GH. Probiotics for the prevention of *Clostridium difficile*-associated diarrhea: a systematic review and meta-analysis. Ann Intern Med. 2012;157:878–88.

Jood S, Khetarpaul N, Goyal R. Efficacy of barley based probiotic food mixture in treatment of pathogenic *E.coli* induced diarrhoea in mice. J Food Sci Technol. 2012;49:200–6.

Kabeerdoss J, Devi RS, Mary RR, Prabhavathi D, Vidya R, Mechenro J, Mahendri NV, Pugazhendhi S, Ramakrishna BS. Effect of yoghurt containing *Bifidobacterium lactis* Bb12® on faecal excretion of secretory immunoglobulin A and human beta-defensin 2 in healthy adult volunteers. Nutr J. 2011;10:138.

Kadooka Y, Sato M, Imaizumi K, Ogawa A, Ikuyama K, Akai Y, Okano M, Kagoshima M, Tsuchida T. Regulation of abdominal adiposity by probiotics (*Lactobacillus gasseri* SBT2055) in adults with obese tendencies in a randomized controlled trial. Eur J Clin Nutr. 2010;64:636–43.

Kadooka Y, Sato M, Ogawa A, Miyoshi M, Uenishi H, Ogawa H, Ikuyama K, Kagoshima M, Tsuchida T. Effect of *Lactobacillus gasseri* SBT2055 in fermented milk on abdominal adiposity in adults in a randomised controlled trial. Br J Nutr. 2013;110:1696–703.

Kailasapathy K, Harmstorf I, Phillips M. Survival of *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in stirred fruit yogurts. LWT Food Sci Technol. 2008;41:1317–22.

Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol. 2006;117:979–987; quiz 988.

Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet. 2001;357:1076–9.

Kalliomäki M, Collado MC, Salminen S, Isolauri E. Early differences in fecal microbiota composition in children may predict overweight. Am J Clin Nutr. 2008;87:534–8.

Kalui CM, Mathara JM, Kutima PM. Probiotic potential of spontaneously fermented cereal based foods-A review. Afr J Biotechnol. 2010;9:2490–8.

Kang E-J, Kim SY, Hwang I-H, Ji Y-J. The effect of probiotics on prevention of common cold: a meta-analysis of randomized controlled trial studies. Korean J Fam Med. 2013;34:2–10.

Karimi R, Mortazavian AM, Karami M. Incorporation of *Lactobacillus casei* in Iranian ultrafiltered Feta cheese made by partial replacement of NaCl with KCl. J Dairy Sci. 2012;95:4209–22.

Karlsson FH, Tremaroli V, Nookaew I, Bergström G, Behre CJ, Fagerberg B, Nielsen J, Bäckhed F. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013;498:99–103.

Kato K, Mizuno S, Umesaki Y, et al. Randomized placebo-controlled trial assessing the effect of *Bifidobacteria*-fermented milk on active ulcerative colitis. Aliment Pharmacol Ther. 2004;20:1133–41.

Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. Nature. 2011;474:327–36.

Kebary KMK, Hussen SA, Badawi RM. Improving viability of *Bifidobacterium* and their effect on frozen ice milk. Egypt J Dairy Sci. 1998;26:319–37.

Kedia G, Vázquez JA, Pandiella SS. Fermentability of whole oat flour, PeriTec flour and bran by *Lactobacillus plantarum*. J Food Eng. 2008;89:246–9.

Kim HJ, Camilleri M, McKinzie S, Lempke MB, Burton DD, Thomforde GM, Zinsmeister AR. A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther. 2003;17:895–904.

Kim T-W, Song H-S, Kim H-Y. Distribution of dominant *Bifidobacteria* in the intestinal microflora of Korean adults and seniors, identified by SDS-PAGE of whole cell proteins and 16S rDNA sequence analysis. J Microbiol Biotechnol. 2005;15:388–94.

Klingberg TD, Budde BB. The survival and persistence in the human gastrointestinal tract of five potential probiotic lactobacilli consumed as freeze-dried cultures or as probiotic sausage. Int J Food Microbiol. 2006;109:157–9.

Klingberg TD, Axelsson L, Naterstad K, Elsser D, Budde BB. Identification of potential probiotic starter cultures for Scandinavian-type fermented sausages. Int J Food Microbiol. 2005;105:419–31.

Kondo S, Xiao J-Z, Satoh T, Odamaki T, Takahashi S, Sugahara H, Yaeshima T, Iwatsuki K, Kamei A, Abe K. Antiobesity effects of *Bifidobacterium breve* strain B-3 supplementation in a mouse model with high-fat diet-induced obesity. Biosci Biotechnol Biochem. 2010;74:1656–61.

Kondo S, Kamei A, Xiao JZ, Iwatsuki K, Abe K. *Bifidobacterium breve* B-3 exerts metabolic syndrome-suppressing effects in the liver of diet-induced obese mice: a DNA microarray analysis. Benef Microbes. 2013a;4:247–51.

Kondo J, Xiao J-Z, Shirahata A, Baba M, Abe A, Ogawa K, Shimoda T. Modulatory effects of *Bifidobacterium longum* BB536 on defecation in elderly patients receiving enteral feeding. World J Gastroenterol. 2013b;19:2162–70.

Kotani Y, Shinkai S, Okamatsu H, et al. Oral intake of *Lactobacillus pentosus* strain b240 accelerates salivary immunoglobulin A secretion in the elderly: a randomized, placebo-controlled, double-blind trial. Immun Ageing. 2010;7:11.

Krishan P, Kumar R, Kumar R. Effect of *Lactobacillus rhamnosus* on anthropometric parameters in obese hyperlipidemic patients. Int J Pharma Recent Res. 2011;3:44–50.

Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. J Allergy Clin Immunol. 2007;119:192–19889.

Kun S, Rezessy-Szabó JM, Nguyen QD, Hoschke Á. Changes of microbial population and some components in carrot juice during fermentation with selected *Bifidobacterium* strains. Process Biochem. 2008;43:816–21.

Kwon H-S, Yang E-H, Lee S-H, Yeon S-W, Kang B-H, Kim T-Y. Rapid identification of potentially probiotic *Bifidobacterium* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol Lett. 2005;250:55–62.

Land MH, Rouster-Stevens K, Woods CR, Cannon ML, Cnota J, Shetty AK. *Lactobacillus* sepsis associated with probiotic therapy. Pediatrics. 2005;115:178–81.

Lavermicocca P. Highlights on new food research. Dig Liver Dis. 2006;38:S295–9.

Lavermicocca P, Lonigro SL, Visconti A, De Angelis M, Valerio F, Morelli L. Table olives containing probiotic microorganisms. Priority date: 5.12.2003 no MI2003A002391. European Patent EP1843664 (granted 8.7.09); 2003.

Lê NT, Champagne CP, Lee BH, Goulet J. Growth of *Lactobacillus paracasei* ssp. *paracasei* on tofu whey. Int J Food Microbiol. 2003;89:67–75.

Lee H-Y, Park J-H, Seok S-H, Baek M-W, Kim D-J, Lee K-E, Paek K-S, Lee Y, Park J-H. Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. Biochim Biophys Acta. 2006;1761:736–44.

Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA. 2005;102:11070–5.

Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124:837–48.

Leyer GJ, Li S, Mubasher ME, Reifer C, Ouwehand AC. Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics. 2009;124:e172–9.

Li B, Tian F, Liu X, Zhao J, Zhang H, Chen W. Effects of cryoprotectants on viability of *Lactobacillus reuteri* CICC6226. Appl Microbiol Biotechnol. 2011;92:609–16.

Lilly DM, Stillwell RH. Probiotics: growth-promoting factors produced by microorganisms. Science. 1965;147:747–8.

Lollo PCB, de Moura CS, Morato PN, et al. Probiotic yogurt offers higher immune-protection than probiotic whey beverage. Food Res Int. 2013;54:118–24.

Lomax AR, Calder PC. Probiotics, immune function, infection and inflammation: a review of the evidence from studies conducted in humans. Curr Pharm Des. 2009;15:1428–518.

Mackay AD, Taylor MB, Kibbler CC, Hamilton-Miller JMT. *Lactobacillus* endocarditis caused by a probiotic organism. Clin Microbiol Infect. 1999;5:290–2.

Macpherson AJ, McCoy KD, Johansen F-E, Brandtzaeg P. The immune geography of IgA induction and function. Mucosal Immunol. 2008;1:11–22.

Madureira AR, Amorim M, Gomes AM, Pintado ME, Malcata FX. Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. Food Res Int. 2011;44:465–70.

Mahdi HA, Tamime AY, Davies G. Some aspects of the production of "Labneh" by ultrafiltration using cow's, sheep's and goat's milk. Egypt J Dairy Sci. 1990;18:345–67.

Mahoney M, Henriksson A. The effect of processed meat and meat starter cultures on gastrointestinal colonization and virulence of Listeria monocytogenes in mice. Int J Food Microbiol. 2003;84:255–61.

Majamaa H, Isolauri E. Probiotics: a novel approach in the management of food allergy. J Allergy Clin Immunol. 1997;99:179–85.

Mann GV. Studies of a surfactant and cholesteremia in the Maasai. Am J Clin Nutr. 1974;27:464–9.

Marafon AP, Sumi A, Alcântara MR, Tamime AY, Nogueira de Oliveira M. Optimization of the rheological properties of probiotic yoghurts supplemented with milk proteins. LWT Food Sci Technol. 2011a;44:511–519.

Marafon AP, Sumi A, Granato D, Alcântara MR, Tamime AY, Nogueira de Oliveira M. Effects of partially replacing skimmed milk powder with dairy ingredients on rheology, sensory profiling, and microstructure of probiotic stirred-type yogurt during cold storage. J Dairy Sci. 2011b;94:5330–5340.

Maragkoudakis P, Nardi T, Bovo B, Corich V, Giacomini A. Valorisation of a milk industry by-product as substrate for microbial growth. J Biotechnol. 2010;150:340.

Martins EMF, Ramos AM, Vanzela ESL, Stringheta PC, de Oliveira Pinto CL, Martins JM. Products of vegetable origin: a new alternative for the consumption of probiotic bacteria. Food Res Int. 2013;51:764–770.

Maruo T, Sakamoto M, Ito C, Toda T, Benno Y. *Adlercreutzia equolifaciens* gen. nov., sp. nov., an equol-producing bacterium isolated from human faeces, and emended description of the genus Eggerthella. Int J Syst Evol Microbiol. 2008;58:1221–7.

Masotti AI, Buckley N, Champagne CP, Green-Johnson J. Immunomodulatory bioactivity of soy and milk ferments on monocyte and macrophage models. Food Res Int. 2011;44:2475–81.

Masson LMP, Rosenthal A, Calado VMA, Deliza R, Tashima L. Effect of ultra-high pressure homogenization on viscosity and shear stress of fermented dairy beverage. LWT Food Sci Technol. 2011;44:495–501.

Matthews DM, Jenks SM. Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. Behav Processes. 2013;96:27–35.

Matthies A, Loh G, Blaut M, Braune A. Daidzein and genistein are converted to equol and 5-hydroxy-equol by human intestinal Slackia isoflavoniconvertens in gnotobiotic rats. J Nutr. 2012;142:40–6.

Mattila-Sandholm T, Myllärinen P, Crittenden R, Mogensen G, Fondén R, Saarela M. Technological challenges for future probiotic foods. Int Dairy J. 2002;12:173–82.
Maukonen J, Mättö J, Kajander K, Mattila-Sandholm T, Saarela M. Diversity and temporal stability of fecal bacterial populations in elderly subjects consuming galacto-oligosaccharide containing probiotic yoghurt. Int Dairy J. 2008;18:386–95.
Messaoudi M, Lalonde R, Violle N, et al. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr. 2011;105:755–64.
Metchnikoff E. The prolongation of life. New York: Arna Press; 1907.
Michail SK, Stolfi A, Johnson T, Onady GM. Efficacy of probiotics in the treatment of pediatric atopic dermatitis: a meta-analysis of randomized controlled trials. Ann Allergy Asthma Immunol. 2008;101:508–16.
Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. Gut. 2004;53:108–14.
Momose Y, Hirayama K, Itoh K. Antagonism of intestinal bacteria isolated from human infants against *Escherichia coli* O157:H7 infection in gnotobiotic mice. Microb Ecol Heal Dis. 2005;17:9–14.
Montalto M, D'Onofrio F, Gallo A, Cazzato A, Gasbarrini G. Intestinal microbiota and its functions. Dig Liver Dis Suppl. 2009;3:30–4.
Morelli L. In vitro selection of probiotic lactobacilli: a critical appraisal. Curr Issues Intest Microbiol. 2000;1:59–67.
Mousavi ZE, Mousavi SM, Razavi SH, Emam-Djomeh Z, Kiani H. Fermentation of pomegranate juice by probiotic lactic acid bacteria. World J Microbiol Biotechnol. 2010;27:123–8.
N'guessan KF, Brou K, Jacques N, Casaregola S, Dje KM. Identification of yeasts during alcoholic fermentation of tchapalo, a traditional sorghum beer from Côte d'Ivoire. Antonie Van Leeuwenhoek. 2011;99:855–64.
Namba K, Yaeshima T, Ishibashi N, Hayasawa H, Yamazaki S. Inhibitory effects of *Bifidobacterium longum* on enterohemorrhagic Escherichia coli O157:H7. Biosci Microflora. 2003;22:85–91.
Namba K, Hatano M, Yaeshima T, Takase M, Suzuki K. Effects of *Bifidobacterium longum* BB536 administration on influenza infection, influenza vaccine antibody titer, and cell-mediated immunity in the elderly. Biosci Biotechnol Biochem. 2010;74:939–45.
Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil. 2011;23(255–264):e119.
Nicolesco CL, Buruleanu LC. Correlation of some substrate parameters in growing *Lactobacillus acidophilus* on vegetable and fruit cocktail juices. Bull UASVM Agric. 2010;67:352–9.
Noorbakhsh R, Yaghmaee P, Durance T. Radiant energy under vacuum (REV) technology: a novel approach for producing probiotic enriched apple snacks. J Funct Foods. 2013;5:1049–56.
Nour M. 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Res Microbiol. 1998;149:433–48.
Nualkaekul S, Salmeron I, Charalampopoulos D. Investigation of the factors influencing the survival of *Bifidobacterium* longum in model acidic solutions and fruit juices. Food Chem. 2011;129:1037–44.
Nualkaekul S, Deepika G, Charalampopoulos D. Survival of freeze dried *Lactobacillus plantarum* in instant fruit powders and reconstituted fruit juices. Food Res Int. 2012a;48:627–33.
Nualkaekul S, Lenton D, Cook MT, Khutoryanskiy VV, Charalampopoulos D. Chitosan coated alginate beads for the survival of microencapsulated *Lactobacillus plantarum* in pomegranate juice. Carbohydr Polym. 2012b;90:1281–7.
O'Mahony L, McCarthy J, Kelly P, et al. *Lactobacillus* and *Bifidobacterium* in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology. 2005;128:541–51.

Odamaki T, Yonezawa S, Kitahara M, Sugahara Y, Xiao J-Z, Yaeshima T, Iwatsuki K, Ohkuma M. Novel multiplex polymerase chain reaction primer set for identification of *Lactococcus* species. Lett Appl Microbiol. 2011a;52:491–6.

Odamaki T, Yonezawa S, Sugahara H, Xiao J, Yaeshima T, Iwatsuki K. A one step genotypic identification of *Lactococcus lactis* subspecies at the species/strain levels. Syst Appl Microbiol. 2011b;34:429–34.

Odamaki T, Sugahara H, Yonezawa S, Yaeshima T, Iwatsuki K, Tanabe S, Tominaga T, Togashi H, Benno Y, Xiao J. Effect of the oral intake of yogurt containing *Bifidobacterium longum* BB536 on the cell numbers of enterotoxigenic *Bacteroides fragilis* in microbiota. Anaerobe. 2012;18:14–8.

Ogata T, Nakamura T, Yaeshima T, Takahashi S, Fukuwatari Y, Ishibashi N, et al. Effect of *Bifidobacterium longum* BB536 administration on the intestinal environment, defecation frequency and fecal characteristics of human volunteers. Biosci Microflora. 1997;16:53–8.

Ogawa M, Shimizu K, Nomoto K, Takahashi M, Watanuki M, Tanaka R, Tanaka T, Hamabata T, Yamasaki S, Takeda Y. Protective effect of *Lactobacillus casei* strain Shirota on Shiga toxin-producing *Escherichia coli* O157:H7 infection in infant rabbits. Infect Immun. 2001;69:1101–8.

Ohishi A, Takahashi S, Ito Y, et al. *Bifidobacterium* septicemia associated with postoperative probiotic therapy in a neonate with omphalocele. J Pediatr. 2010;156:679–81.

Olivares C, Solano F. New insights into the active site structure and catalytic mechanism of tyrosinase and its related proteins. Pigment Cell Melanoma Res. 2009;22:750–60.

Olivares M, Díaz-Ropero MP, Sierra S, Lara-Villoslada F, Fonollá J, Navas M, Rodríguez JM, Xaus J. Oral intake of *Lactobacillus fermentum* CECT5716 enhances the effects of influenza vaccination. Nutrition (Burbank, Los Angeles County, California). 2007;23:254–60.

Oliveira RPDS, Perego P, Converti A, De Oliveira MN. Effect of inulin on growth and acidification performance of different probiotic bacteria in co-cultures and mixed culture with *Streptococcus thermophilus*. J Food Eng. 2009;91:133–9.

Oliveira RP de S, Perego P, Oliveira MN de, Converti A. Effect of inulin as prebiotic and synbiotic interactions between probiotics to improve fermented milk firmness. J Food Eng. 2011;107:36–40.

Olson DW, Aryana KJ. An excessively high *Lactobacillus acidophilus* inoculation level in yogurt lowers product quality during storage. LWT Food Sci Technol. 2008;41:911–8.

Ortiz-Lucas M, Tobías A, Saz P, Sebastián JJ. Effect of probiotic species on irritable bowel syndrome symptoms: a bring up to date meta-analysis. Rev Espanola Enfermedades Dig Organo Soc Espanola Patol Dig. 2013;105:19–36.

Ou C, Kuo H, Wang L, Hsu T, Chuang H, Liu C, Chang J, Yu H, Yang KD. Prenatal and postnatal probiotics reduces maternal but not childhood allergic diseases: a randomized, double-blind, placebo-controlled trial. Clin Exp Allergy. 2012;42:1386–96.

Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. 2011;11:85–97.

Ouwehand AC, Ten Bruggencate SJM, Schonewille AJ, Alhoniemi E, Forssten SD, Bovee-Oudenhoven IMJ. *Lactobacillus acidophilus* supplementation in human subjects and their resistance to enterotoxigenic *Escherichia coli* infection. Br J Nutr. 2013; 12:1–9.

Park Y, Albright KJ, Liu W, Storkson JM, Cook ME, Pariza MW. Effect of conjugated linoleic acid on body composition in mice. Lipids. 1997;32:853–8.

Park MS, Kim MJ, Ji GE. Assessment of lipopolysaccharide-binding activity of *Bifidobacterium* and its relationship with cell surface hydrophobicity, autoaggregation, and inhibition of interleukin-8 production. J Microbiol Biotechnol. 2007;17:1120–6.

Parker RB. Probiotics, the other half of the antibiotic story. Anim Nutr Heal. 1974;29:4–8.

Patel HM, Wang R, Chandrashekar O, Pandiella SS, Webb C. Proliferation of *Lactobacillus plantarum* in solid-state fermentation of oats. Biotechnol Prog. 2004;20:110–6.

Pelucchi C, Chatenoud L, Turati F, Galeone C, Moja L, Bach J-F, La Vecchia C. Probiotics supplementation during pregnancy or infancy for the prevention of atopic dermatitis: a meta-analysis. Epidemiol Camb Mass. 2012;23:402–14.

Peng G-C, Hsu C-H. The efficacy and safety of heat-killed *Lactobacillus paracasei* for treatment of perennial allergic rhinitis induced by house-dust mite. Pediatr Allergy Immunol. 2005;16:433–8.

Pennacchia C, Vaughan EE, Villani F. Potential probiotic *Lactobacillus* strains from fermented sausages: further investigations on their probiotic properties. Meat Sci. 2006;73:90–101.

Pereira ALF, Maciel TC, Rodrigues S. Probiotic beverage from cashew apple juice fermented with *Lactobacillus casei*. Food Res Int. 2011;44:1276–83.

Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M. Folate production by *Bifidobacteria* as a potential probiotic property. Appl Environ Microbiol. 2007;73:179–85.

Possemiers S, Marzorati M, Verstraete W, Van de Wiele T. Bacteria and chocolate: a successful combination for probiotic delivery. Int J Food Microbiol. 2010;141:97–103.

Prantera C, Scribano ML, Falasco G, Andreoli A, Luzi C. Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomised controlled trial with *Lactobacillus* GG. Gut. 2002;51:405–9.

Prescott SL, Wiltschut J, Taylor A, Westcott L, Jung W, Currie H, Dunstan JA. Early markers of allergic disease in a primary prevention study using probiotics: 2.5-year follow-up phase. Allergy. 2008;63:1481–90.

Pyar H, Peh K-K. Effect of cryoprotective agents on survival and stability of *Lactobacillus acidophilus* cultured in food-grade medium. Int J Dairy Technol. 2011;64:578–84.

Radulović Z, Živković D, Mirković N, Petrušić M, Stajić S, Perunović M, Paunović D. Effect of probiotic bacteria on chemical composition and sensory quality of fermented sausages. Procedia Food Sci. 2011;1:1516–22.

Ranadheera RDCS, Baines SK, Adams MC. Importance of food in probiotic efficacy. Food Res Int. 2010;43:1–7.

Rathore S, Salmerón I, Pandiella SS. Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. Food Microbiol. 2012;30:239–44.

Rautava S, Kainonen E, Salminen S, Isolauri E. Maternal probiotic supplementation during pregnancy and breast-feeding reduces the risk of eczema in the infant. J Allergy Clin Immunol. 2012;130:1355–60.

Rautio M, Jousimies-Somer H, Kauma H, Pietarinen I, Saxelin M, Tynkkynen S, Koskela M. Liver abscess due to a *Lactobacillus rhamnosus* strain indistinguishable from *L. rhamnosus* strain GG. Clin Infect Dis. 1999;28:1159–60.

Rivera-Espinoza Y, Gallardo-Navarro Y. Non-dairy probiotic products. Food Microbiol. 2010;27:1–11.

Rodrigues D, Rocha-Santos TAP, Pereira CI, Gomes AM, Malcata FX, Freitas AC. The potential effect of FOS and inulin upon probiotic bacterium performance in curdled milk matrices. LWT - Food Sci Technol. 2011;44:100–8.

Rose MA, Stieglitz F, Köksal A, Schubert R, Schulze J, Zielen S. Efficacy of probiotic *Lactobacillus* GG on allergic sensitization and asthma in infants at risk. Clin Exp Allergy. 2010;40:1398–405.

Rossi M, Amaretti A, Raimondi S. Folate production by probiotic bacteria. Nutrients. 2011;3:118–34.

Rößle C, Auty MAE, Brunton N, Gormley RT, Butler F. Evaluation of fresh-cut apple slices enriched with probiotic bacteria. Innov Food Sci Emerg Technol. 2010a;11:203–9.

Rößle C, Brunton N, Gormley RT, Ross PR, Butler F. Development of potentially synbiotic fresh-cut apple slices. J Funct Foods. 2010b;2:245–54.

Roy D. Technological aspects related to the use of *Bifidobacteria* in dairy products. Le Lait. 2005;85:39–56.

Rubio R, Aymerich T, Bover-Cid S, Guàrdia MD, Arnau J, Garriga M. Probiotic strains *Lactobacillus plantarum* 299 V and *Lactobacillus rhamnosus* GG as starter cultures for fermented sausages. LWT - Food Sci Technol. 2013;54:51–6.

Ruiz-Moyano S, Martín A, Benito MJ, Nevado FP, de Guía Córdoba M. Screening of lactic acid bacteria and *Bifidobacteria* for potential probiotic use in Iberian dry fermented sausages. Meat Sci. 2008;80:715–721.

Ruiz-Moyano S, Martín A, Benito MJ, Hernández A, Casquete R, de Guia Córdoba M. Application of *Lactobacillus fermentum* HL57 and *Pediococcus acidilactici* SP979 as potential probiotics in the manufacture of traditional Iberian dry-fermented sausages. Food Microbiol. 2011;28:839–847.

Saarela M, Virkajärvi I, Nohynek L, Vaari A, Mättö J. Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. Int J Food Microbiol. 2006;112:171–8.

Saarela M, Alakomi HL, Mättö J, Ahonen AM, Puhakka A, Tynkkynen S. Improving the storage stability of *Bifidobacterium breve* in low pH fruit juice. Int J Food Microbiol. 2011;149:106–10.

Said HM, Mohammed ZM. Intestinal absorption of water-soluble vitamins: an update. Curr Opin Gastroenterol. 2006;22:140–6.

Salminen S, von Wright A, Morelli L, et al. Demonstration of safety of probiotics: a review. Int J Food Microbiol. 1998;44:93–106.

Sánchez B, de los Reyes-Gavilán CG, Margolles A, Gueimonde M. Probiotic fermented milks: Present and future. Int J Dairy Technol. 2009;62:472–483.

Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology. 2004;126:1620–33.

Sarvan I, Valerio F, Lonigro SL, de Candia S, Verkerk R, Dekker M, Lavermicocca P. Glucosinolate content of blanched cabbage (Brassica oleracea var. capitata) fermented by the probiotic strain *Lactobacillus paracasei* LMG-P22043. Food Res Int. 2013;54:706–10.

Savard P, Lamarche B, Paradis M-E, Thiboutot H, Laurin É, Roy D. Impact of *Bifidobacterium animalis* subsp. *lactis* BB-12 and, *Lactobacillus acidophilus* LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. Int J Food Microbiol. 2011;149:50–7.

SCAN. Opinion of the scientific committee on animal nutrition on the safety of use of *Bacillus* species in animal nutrition. European Commission, health and consumer protection directorate-general (SCAN) scientific committee on animal nutrition. 2000; http://ec.europa.eu/food/fs/sc/scan/out41_en.pdf

Schillinger U, Guigas C, Heinrich Holzapfel W. In vitro adherence and other properties of lactobacilli used in probiotic yoghurt-like products. Int Dairy J. 2005;15:1289–1297.

Schultz M, Timmer A, Herfarth HH, Sartor RB, Vanderhoof JA, Rath HC. *Lactobacillus* GG in inducing and maintaining remission of Crohn's disease. BMC Gastroenterol. 2004;4:5.

Sekirov I, Russell SL, Antunes LCM, Finlay BB. Gut microbiota in health and disease. Physiol Rev. 2010;90:859–904.

Senaka Ranadheera C, Evans CA, Adams MC, Baines SK. Probiotic viability and physico-chemical and sensory properties of plain and stirred fruit yogurts made from goat's milk. Food Chem. 2012;135:1411–8.

Sendra E, Fayos P, Lario Y, Fernández-López J, Sayas-Barberá E, Pérez-Alvarez JA. Incorporation of citrus fibers in fermented milk containing probiotic bacteria. Food Microbiol. 2008;25:13–21.

Serrano-Niño JC, Cavazos-Garduño A, Hernandez-Mendoza A, Applegate B, Ferruzzi MG, San Martin-González MF, García HS. Assessment of probiotic strains ability to reduce the bioaccessibility of aflatoxin M1 in artificially contaminated milk using an in vitro digestive model. Food Control. 2013;31:202–207.

Settanni L, van Sinderen D, Rossi J, Corsetti A. Rapid differentiation and in situ detection of 16 sourdough *Lactobacillus* species by multiplex PCR. Appl Environ Microbiol. 2005;71:3049–59.

Shah NP. Functional foods from probiotics and prebiotics. Food Technol. 2001;55:46–53.

Shah NP, Ravula RR. Influence of water activity on fermentation, organic acids production and viability of yogurt and probiotic bacteria. Aust J Dairy Technol. 2000a;55:127–31.

Shah NP, Ravula RR. Microencapsulation of probiotic bacteria and their survival in frozen fermented dairy desserts. Aust J Dairy Technol. 2000b;55:139–44.

Sharp MD, McMahon DJ, Broadbent JR. Comparative Evaluation of Yogurt and Low-Fat Cheddar Cheese as Delivery Media for Probiotic *Lactobacillus casei*. J Food Sci. 2008;73:M375–7.

Sheehan VM, Ross P, Fitzgerald GF. Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. Innov Food Sci Emerg Technol. 2007;8:279–84.

Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, Bae JW. An increase in the *Akkermansia* spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut. 2013. doi: 10.1136/gutjnl-2012-303839

Sidira M, Saxami G, Dimitrellou D, Santarmaki V, Galanis A, Kourkoutas Y. Monitoring survival of *Lactobacillus casei* ATCC 393 in probiotic yogurts using an efficient molecular tool. J Dairy Sci. 2013;96:3369–77.

Siener R, Bangen U, Sidhu H, Hönow R, von Unruh G, Hesse A. The role of *Oxalobacter formigenes* colonization in calcium oxalate stone disease. Kidney Int. 2013;83:1144–9.

Simpson PJ, Stanton C, Fitzgerald GF, Ross RP. Intrinsic tolerance of *Bifidobacterium* species to heat and oxygen and survival following spray drying and storage. J Appl Microbiol. 2005;99:493–501.

Sokol H, Pigneur B, Watterlot L, et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci USA. 2008;105:16731–6.

Srisuvor N, Chinprahast N, Prakitchaiwattana C, Subhimaros S. Effects of inulin and polydextrose on physicochemical and sensory properties of low-fat set yoghurt with probiotic-cultured banana purée. LWT Food Sci Technol. 2013;51:30–6.

Stadnik J, Dolatowski ZJ. Biogenic amines content during extended ageing of dry-cured pork loins inoculated with probiotics. Meat Sci. 2012;91:374–7.

Stadnik J, Dolatowski ZJ. Changes in selected parameters related to proteolysis during ageing of dry-cured pork loins inoculated with probiotics. Food Chem. 2013;139:67–71.

Stanton C, Fitzgerald G, Paul Ross R, Desmond C, Coakley M, Kevin Collins J. Challenges facing development of probiotic-containing functional foods. In: Farnworth E, editor. Handbook of fermented functional foods. England: CRC Press; 2003. pp. 27–58.

Strachan DP. Hay fever, hygiene, and household size. BMJ. 1989;299:1259–60.

Sul SY, Kim HJ, Kim TW, Kim HY. Rapid identification of *Lactobacillus* and *Bifidobacterium* in probiotic products using multiplex PCR. J Microbiol Biotechnol. 2007;17:490–5.

Szajewska H, Mrukowicz JZ. Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: a systematic review of published randomized, double-blind, placebo-controlled trials. J Pediatr Gastroenterol Nutr. 2001;33(Suppl 2):S17–25.

Takahashi T, Nakagawa E, Nara T, Yajima T, Kuwata T. Effects of orally ingested *Bifidobacterium longum* on the mucosal IgA response of mice to dietary antigens. Biosci Biotechnol Biochem. 1998;62:10–5.

Takeda K, Okumura K. Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the human NK-cell activity. J Nutr. 2007;137:791S–3S.

Takeda Y, Nakase H, Namba K, Inoue S, Ueno S, Uza N, Chiba T. Upregulation of T-bet and tight junction molecules by *Bifidobacterium longum* improves colonic inflammation of ulcerative colitis. Inflamm Bowel Dis. 2009;15:1617–8.

Talwalkar A, Kailasapathy K. A review of oxygen toxicity in probiotic yogurts: influence on the survival of probiotic bacteria and protective techniques. Compr Rev Food Sci Food Saf. 2004;3:117–24.

Tamime AY. Yoghurt: science and technology. 2nd ed. Boca Raton, Cambridge: CRC Press, Woodhead Pub; 1999.

Tamime AY, Wszolek M, Božanić R, Özer B. Popular ovine and caprine fermented milks. Small Rumin Res. 2011;101:2–16.

Taverniti V, Guglielmetti S. The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: proposal of paraprobiotic concept). Genes Nutr. 2011;6:261–74.

Taylor AL, Dunstan JA, Prescott SL. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. J Allergy Clin Immunol. 2007;119:184–91.

Tejada-Simon MV, Lee JH, Ustunol Z, Pestka JJ. Ingestion of yogurt containing *Lactobacillus acidophilus and Bifidobacterium* to potentiate immunoglobulin A responses to cholera toxin in mice. J Dairy Sci. 1999;82:649–60.

Tillisch K, Labus JS, Ebrat B, Stains J, Naliboff B, Guyonnet D, Legrain-Raspaud S, Trotin B, Mayer EA. Modulation of the brain-gut axis after 4-week intervention with a probiotic fermented dairy product. Gastroenterology. 2012;142:S–115.

Tillisch K, Labus J, Kilpatrick L, et al. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology. 2013;144:1394–1401, 1401.e1–4.

Tilsala-Timisjärvi A, Alatossava T. Development of oligonucleotide primers from the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Int J Food Microbiol. 1997;35:49–56.

Tissier H. Repartition des microbes dans l'intestin du nourisson. Ann Inst Pasteur Paris. 1905;19:109–23.

Toprak NU, Yagci A, Gulluoglu BM, Akin ML, Demirkalem P, Celenk T, Soyletir G. A possible role of *Bacteroides fragilis* enterotoxin in the aetiology of colorectal cancer. Clin Microbiol Infect. 2006;12:782–6.

Tripolt NJ, Leber B, Blattl D, et al. Short communication: effect of supplementation with *Lactobacillus casei* Shirota on insulin sensitivity, β-cell function, and markers of endothelial function and inflammation in subjects with metabolic syndrome a pilot study. J Dairy Sci. 2013;96:89–95.

Tsai JS, Lin YS, Pan BS, Chen TJ. Antihypertensive peptides and γ-aminobutyric acid from prozyme 6 facilitated lactic acid bacteria fermentation of soymilk. Process Biochem. 2006;41:1282–8.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444:1027–31.

Uysal-Pala C, Karagul-Yuceer Y, Pala A, Savas T. Sensory properties of drinkable yogurt made from milk of different goat breeds. J Sens Stud. 2006;21:520–33.

Van der Aa LB, van Aalderen WMC, Heymans HSA, Henk Sillevis Smitt J, Nauta AJ, Knippels LMJ, Ben Amor K, Sprikkelman AB, Synbad Study Group. Synbiotics prevent asthma-like symptoms in infants with atopic dermatitis. Allergy. 2011;66:170–177.

Van Tongeren SP, Slaets JPJ, Harmsen HJM, Welling GW. Fecal microbiota composition and frailty. Appl Environ Microbiol. 2005;71:6438–42.

Verdú EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. Gut. 2006;55:182–90.

Vesterlund S, Salminen K, Salminen S. Water activity in dry foods containing live probiotic bacteria should be carefully considered: a case study with *Lactobacillus rhamnosus* GG in flaxseed. Int J Food Microbiol. 2012;157:319–21.

Vinderola G, Prosello W, Molinari F, Ghiberto D, Reinheimer J. Growth of *Lactobacillus paracasei* A13 in Argentinian probiotic cheese and its impact on the characteristics of the product. Int J Food Microbiol. 2009;135:171–4.

Vliagoftis H, Kouranos VD, Betsi GI, Falagas ME. Probiotics for the treatment of allergic rhinitis and asthma: systematic review of randomized controlled trials. Ann Allergy Asthma Immunol. 2008;101:570–9.

Wang X, Brown IL, Evans AJ, Conway PL. The protective effects of high amylose maize (amylomaize) starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract. J Appl Microbiol. 1999;87:631–9.

Wang MF, Lin HC, Wang YY, Hsu CH. Treatment of perennial allergic rhinitis with lactic acid bacteria. Pediatr Allergy Immunol. 2004;15:152–8.

Wang XL, Shin KH, Hur HG, Kim SI. Enhanced biosynthesis of dihydrodaidzein and dihydrogenistein by a newly isolated bovine rumen anaerobic bacterium. J Biotechnol. 2005;115:261–9.

Wang J, Guo Z, Zhang Q, Yan L, Chen W, Liu XM, Zhang HP. Fermentation characteristics and transit tolerance of probiotic *Lactobacillus casei* Zhang in soymilk and bovine milk during storage. J Dairy Sci. 2009;92:2468–76.

Wang W, Bao Y, Hendricks GM, Guo M. Consistency, microstructure and probiotic survivability of goats' milk yoghurt using polymerized whey protein as a co-thickening agent. Int Dairy J. 2012a;24:113–9.

Wang Y, Xie J, Wang N, Li Y, Sun X, Zhang Y, Zhang H. *Lactobacillus casei* Zhang modulate cytokine and toll-like receptor expression and beneficially regulate PolyI:C-induced immune responses in RAW264.7 macrophages. Microbiol Immunol. 2012b. doi: 10.1111/j.1348-0421.2012.00516.x

Wang Y, Li Y, Xie J, Zhang Y, Wang J, Sun X, Zhang H. Protective effects of probiotic *Lactobacillus casei* Zhang against endotoxin- and d-galactosamine-induced liver injury in rats via anti-oxidative and anti-inflammatory capacities. Int Immunopharmacol. 2013;15:30–7.

Ward LJ, Timmins MJ. Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. Lett Appl Microbiol. 1999;29:90–2.

Wei YX, Zhang ZY, Liu C, Malakar PK, Guo XK. Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences. World J Gastroenterol. 2012;18:479–88.

West CE, Hammarström M-L, Hernell O. Probiotics during weaning reduce the incidence of eczema. Pediatr Allergy Immunol. 2009;20:430–7.

West CE, Hammarström ML, Hernell O. Probiotics in primary prevention of allergic disease: follow-up at 8–9 years of age. Allergy. 2013;68:1015–20.

Wickens K, Black P, Stanley TV, Mitchell E, Barthow C, Fitzharris P, Purdie G, Crane J. A protective effect of *Lactobacillus rhamnosus* HN001 against eczema in the first 2 years of life persists to age 4 years. Clin Exp Allergy. 2012;42:1071–9.

Wickens K, Stanley TV, Mitchell EA, Barthow C, Fitzharris P, Purdie G, Siebers R, Black PN, Crane J. Early supplementation with *Lactobacillus rhamnosus* HN001 reduces eczema prevalence to 6 years: does it also reduce atopic sensitization? Clin Exp Allergy. 2013;43:1048–57.

Wu S, Rhee K-J, Albesiano E, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009a;15:1016–22.

Wu R, Wang L, Wang J, Li H, Menghe B, Wu J, Guo M, Zhang H. Isolation and preliminary probiotic selection of lactobacilli from koumiss in Inner Mongolia. J Basic Microbiol. 2009b;49:318–26.

Xiao JZ, Kondo S, Takahashi N, Miyaji K, Oshida K, Hiramatsu A, Iwatsuki K, Kokubo S, Hosono A. Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. J Dairy Sci. 2003;86:2452–61.

Xiao JZ, Kondo S, Yanagisawa N, et al. Probiotics in the treatment of Japanese cedar pollinosis: a double-blind placebo-controlled trial. Clin Exp Allergy. 2006;36:1425–35.

Xiao JZ, Kondo S, Yanagisawa N, Miyaji K, Enomoto K, Sakoda T, Iwatsuki K, Enomoto T. Clinical efficacy of probiotic *Bifidobacterium longum* for the treatment of symptoms of Japanese cedar pollen allergy in subjects evaluated in an environmental exposure unit. Allergol Int. 2007;56:67–75.

Ya T, Zhang Q, Chu F, Merritt J, Bilige M, Sun T, Du R, Zhang H. Immunological evaluation of *Lactobacillus casei* Zhang: a newly isolated strain from koumiss in Inner Mongolia. China. BMC Immunol. 2008;9:68.

Yaeshima T, Takahashi S, Matsumoto N, Ishibashi N, Hayasawa H, Iino H. Effect of yogurt containing *Bifidobacterium longum* BB536 on the intestinal environment, fecal characteristics and defecation frequency: a comparison with standard yogurt. Biosci Microflora. 1997;16:73–7.

Yeon SW, You YS, Kwon HS, Yang EH, Ryu JS, Kang BH, Kang JH. Fermented milk of *Lactobacillus helveticus* IDCC3801 reduces beta-amyloid and attenuates memory deficit. J Funct Foods. 2010;2:143–52.

Yoshimura K, Matsui T, Itoh K. Prevention of Escherichia coli O157:H7 infection in gnotobiotic mice associated with *Bifidobacterium* strains. Antonie Van Leeuwenhoek. 2010;97:107–17.

Zare F, Champagne CP, Simpson BK, Orsat V, Boye JI. Effect of the addition of pulse ingredients to milk on acid production by probiotic and yoghurt starter cultures. LWT Food Sci Technol. 2012;45:155–60.

Zhang Y, Wang L, Zhang J, Li Y, He Q, Li H, Guo X, Guo J, Zhang H. Probiotic *Lactobacillus casei* Zhang ameliorates high-fructose-induced impaired glucose tolerance in hyperinsulinemia rats. Eur J Nutr. 2013;. doi:10.1007/s00394-013-0519-5

Zhong Z, Zhang W, Du R, Meng H, Zhang H. *Lactobacillus casei* Zhang stimulates lipid metabolism in hypercholesterolemic rats by affecting gene expression in the liver. Eur J Lipid Sci Technol. 2012;114:244–52.

Zubaidah E, Nurcholis M, Wulan SN, Kusuma A. Comparative study on synbiotic effect of fermented rice bran by probiotic lactic acid bacteria *Lactobacillus casei* and newly isolated *Lactobacillus plantarum* B2 in wistar rats. APCBEE Procedia. 2012;2:170–7.

Chapter 6
Lactic Acid Bacteria and the Human Gastrointestinal Tract

Lai-yu Kwok

Abstract The human gastrointestinal tract is composed of complex ecological environments, which hosts a high number and diversity of gut microorganisms, mainly including bacteria, archaea, fungi, and viruses. The advent of the next-generation DNA sequencing technology has transformed our current knowledge and understanding of the structure and function of the human gut microbiota. Some lactic acid bacteria and bifidobacteria are common inhabitants of the human gut, and they are believed to hold strong link to a healthy gastrointestinal tract by exhibiting probiotic functions. Therefore, many of these members have also been tested in human trials for their clinical beneficial effects and their potential to be developed as nutraceutical products. On the other hand, some other members have posed emerging concerns in clinical antibiotic resistance and atypical infection in rare occasions. Therefore, a careful balance should be struck in the design of policies on their applications. The study of the human gut metagenomes will definitely remain as a major focus in life and medical sciences in the twenty-first century. This chapter reviews the current status in this research area, in particular, our knowledge in the metagenomics of the human gastrointestinal tract, as well as the relationship between lactic acid bacteria and the human gut. Current limitations and challenges of these research areas are further discussed.

Keywords Lactic acid bacteria · Human gastrointestinal tract · Metagenomics

L. Kwok (✉)
Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education,
Inner Mongolia Agricultural University, Hohhot, China
e-mail: kwok_ly@yahoo.com

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_6,
© Springer Science+Business Media Dordrecht 2014

6.1 Metagenomics of the Human Gastrointestinal Tract

6.1.1 Background of Metagenomics Study of the Human Body

Metagenomics is a relatively new area within the field. The term "metagenomics" was coined by Handelsman et al. (1998), and it is defined as the direct genetic analysis of the genome of an environmental sample. Microbial genomics has flourished since the publication of the first bacterial genome of *Haemophilus influenza* (Fleischmann et al. 1995). However, the traditional microbial genomic approach is restricted to the sequencing of only one single microorganism at a time, and it often requires the cultivation of the target microbe. It has been estimated that over 99 % of the world microbial populations are not cultivatable in laboratory (Kaeberlein et al. 2002). The metagenomic approach does not only solve the barrier of culturability, but also enables a thorough description of the yet uncultivable microbial members in any habitat.

With the advent of the next-generation sequencing (NGS) technology and the large reduction in the cost of DNA sequencing, there is a growing trend of using such metagenomic approach in studying the microbial populations in diverse habitats. The generated data would give meaningful information on the microbial interaction at community level, as well as novel implications on the overall microbial function. The application of metagenomic approach has rapidly been extended to the study of the human microbiome, including the airways, gastrointestinal tract (GIT), oral cavities, skin, urogenital tract, and other body sites (Markowitz et al. 2012). Among the various studied body sites, the human GIT has received extensive attention, as the microbiota of the GIT plays crucial roles in health maintenance and balance (Robles Alonso and Guarner 2013; Aziz et al. 2013; Power et al. 2013). The human gut microbiota is composed of approximately 10^{11-12} microorganisms/gram of fecal content (i.e., over 10 times of the total cell number of a normal human), of which 95 % are anaerobes (Ley et al. 2006). In the case of an unbalanced health conditions or external perturbations like antibiotic administration or immunosuppressive therapy, gut dysbiosis may occur. It is now known that gut dysbiosis is linked to a number of medical conditions like irritable bowel syndrome (IBS), rheumatoid arthritis, inflammatory bowel disease, obesity, and diabetes (Manichanh et al. 2012; Hawrelak and Myers 2004; Stecher et al. 2013).

Much ongoing effort has focused on characterizing the human gut metagenomes. Some of the major objectives of such projects are to establish a human "core gut microbiota" gene catalog, to develop signatures and biomarkers at individual level (e.g., people of different age group, from different culture/region, or having specific conditions like obesity and diabetes), and to understand the overall function of the gut microbiota. Such knowledge may help to determine the disease targets for early diagnosis and prevention, as well as to provide fundamental understanding on the roles of the complex gut microbial communities in health maintenance.

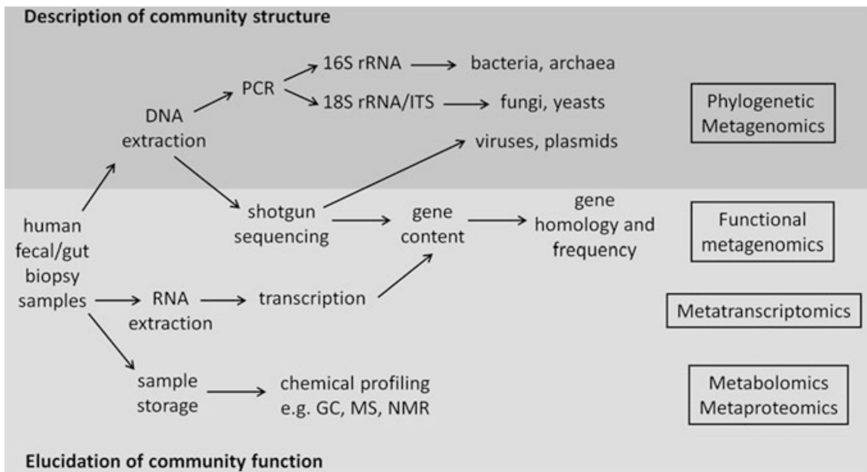


Fig. 6.1 The "omics" approach in studying human GIT microbiome

6.1.2 High Throughput Sequencing in Metagenomics

Prior to the NGS technology, more classical molecular biology methods have been used to study the microbiota composition in the GIT and other body sites. In situ hybridization, quantitative real-time polymerase chain reaction (qPCR), and phylogenetic microarray have been used extensively, as they are able to provide quantitative data and are of high sensitivity. However, these methods are limited in their application to only known organisms. PCR-denaturing gradient gel electrophoresis (DGGE) coupled to clone library is another commonly used approach, but it is only semiquantitative, time-consuming, and restrictive to the detection of highly abundant groups.

During the last few years, the newly developed NGS technology has started to replace the traditionally used Sanger sequencing method in the study of gut metagenomics. The major advantages of NGS technology are the low cost, and the relatively direct sequencing enables the skipping of the laborious steps of cloning and cultivation of the individual microbes (see Fig. 6.1). The NGS phylogenetic metagenomic approach is mainly based on a first PCR to amplify a specific region of the 16S rRNA gene (for bacteria and archaea) or 18S rRNA/internal transcribed spacer (ITS) region (for fungi). The main platforms which have so far been used for NGS are the Genome Sequencer 454 FLX and the Illumina Genome Analyzer systems. The Genome Sequencer 454 FLX system could generate around 400,000 reads of 250–350 base pair length on average, whereas the Illumina Genome Analyzer system produced reads of 35–75 base pair (Maccaferri et al. 2011). However, the latter platform was thought to be not reliable enough for both "taxonomic classification" and "community diversity analysis" (Maccaferri et al. 2011; Claesson et al. 2010).

6.1.3 Gut Reference Genomes and Gene Catalogs

Large-scale metagenomic approach-based projects for studying the human gut microbiomes started to produce an enormous amount of data (see Table 6.1 for a summary of the main research programs of the International Human Microbiome Consortium). One of the principal objectives of these projects is to provide reference information on the gut microbial species and gene makeup, which give insights into their function.

The BGI Sequencing Center in China together with the EU Metagenomics of the Human Intestinal Tract (MetaHIT) consortium finished a large-scale metagenomic study, which analyzed samples from 124 European individuals, sequenced 576.7 gigabases in total, and covered at least 85 % the cohort's gene pool (Qin et al. 2010). Approximately, 3.3 million nonredundant microbial genes (about 150 times the human proteome) were found, of which >99 % were of bacterial origin. Around 40 % of the genes from each subject were shared by at least half of the studied cohort, and that <10 % were shared by more than half of the participants. The study also tried to define a minimal gut microbiome genome in terms of functions shared between most individuals. Around 75 % of uncharacterized orthologous groups and/or novel gene families constituted the vital gut functionalities, and the biological role of around 80 % of minimal gut metagenome clusters were undefined. Results from Qin et al. further reported the existence of between 1,000 and 1,150 prevalent bacterial species within the complete cohort, and each person had at least 160. These genes and species were largely shared between individuals, but the species abundance varied highly from person to person (between 12- and 2187-fold).

Another major sequencing effort, the US Human Microbiome Project (HMP) funded by the National Institutes of Health, has contributed impressive input in creating a catalog of reference genome associated with health and disease. The initial phase of the project sequenced and annotated 178 microbial reference genomes (including 151 from human gut) completely. 29,987 out of the total 547,968 analyzed predicted polypeptides were considered as novel, based on the criteria used in the study (i.e., unmasked sequence length >100 amino acids; no BLASTP match to any nonreference entry) (Nelson et al. 2010). Further, work of the US HMP sequenced 4,788 specimens collected from 18 (women) and 15 (men) body sites, respectively, from 242 healthy individuals (Human Microbiome Project Consortium 2012). Fecal specimens were also collected for 16S rRNA pyrosequencing and functional metagenomic analyses. Results from this study are consistent with previous ones that there was a high inter-body site variability of microbiome composition within the same individual, and that the diversity and abundance of each habitat's signature microbes varied widely even among healthy subjects. Factors like diet, host biometric features, genetic background, nutritional status, underlying diseases, and metabolic conditions, may play important roles in shaping the diversity and variability of the human gut microbiome (see Table 6.2 for a review of recently performed metagenomics studies).

Table 6.1 Major international consortia involving in the study of the human microbiome

Research program	Year	Funding source	Principal participating institutes	Program goals and website
NIH Jumpstart	2007–2008	NIH	Sequencing centers at Baylor College of Medicine, The Broad Institute, The J. Craig Venter Institute, Washington University School of Medicine (USA)	To generate complete genome sequences of 200 bacterial strains isolated from human body; to perform 16S rRNA metagenomic sequence analysis of 5 selected sampled body sites
ELDERMET Project	2007–2013	National Development Food Research Health Initiative and Science Foundation Ireland	University College Cork, Cork University Hospital, and Teagasc, Moorepark (Republic of Ireland)	To characterize the fecal microbiota associated with aging; to correlate fecal microbial metagenome with health, diet, and lifestyle, in terms of microbial diversity, composition and metabolic potential http://eldermet.ucc.ie/
Metagenomics of the Human Intestinal Tract (MetaHIT)	2008–2011	European Commission	INRA, CEA Genoscope, Danone (France); EMBL (Germany); HUVH (Spain); European Institute of Oncology (Italy); Technical University of Denmark, Novo Nordisk Foundation Centre, SDU (Denmark); UCB Pharma, Belgium; Wageningen University (Netherlands); WTSI (UK); BGI (China)	To describe the role of microbiota in inflammatory bowel disease (IBD) and obesity; and to generate a reference catalog of intestinal microbial genes. http://www.metahit.eu

(continued)

Table 6.1 (continued)

Research program	Year	Funding source	Principal participating institutes	Program goals and website
Microbes, Human Intestinal Microbiome in Obesity and Nutritional Transition	2008–2010	French National Agency for Research (in part)	INRA, UPMC, CEA Genoscope (France)	To identify metagenomic signatures that characterize the relationship between the intestinal microbiota and the nutritional/metabolic status of the host. http://www.inra.fr/micro_obes_eng/
NIH Human Microbiome Project	2008–2012	NIH Roadmap Program	NIH (USA)	To extensively characterize microbes living in/on human body; and study whether there are correlations between human microbiome and health. http://nihroadmap.nih.gov/hmp/
Data Analysis and Coordination Center (DACC)	2008–2013	NIH Human Microbiome Project (HMP)	Institute for Genome Sciences (USA)	To assist in standardization of data pipelines including storage, analysis and display of data; and to provide access to data. http://www.hmpdacc.org
The Australian Jumpstart Human Microbiome Project	From 2009	CSIRO	CSIRO (Australia)	To sequence specific bacterial strains and apply metagenomics techniques to investigate the interaction between intestinal microbes and their host

(continued)

Table 6.1 (continued)

Research program	Year	Funding source	Principal participating institutes	Program goals and website
Canadian Human Microbiome Initiative	From 2009	Canadian Institutes of Health Research	University of Guelph, University of British Columbia, University Health Network, University of Alberta, McGill University, University of Waterloo, Ontario, University of Calgary, University of Ottawa (Canada)	To perform the major projects: "Investigating the potential effects of host-derived stress hormones on the human gut microflora'; "The role of the gastrointestinal microbiota in asthma"; "Linking infectious agents to cancer : a metagenomics approach"; "Assessing the impact of polymicrobial pulmonary infections in cystic fibrosis via metagenomics"; "The impact of antibiotics on intestinal microbiota of infants"; "Microbial metagenomics of the intestinal microbiota and the etiology of Clostridium difficile—associated disease in hospitalized patients"; "Metagenomics characterization of the human vaginal microbiome"; "Establishing a complete taxonomic baseline for the human microbiome"; "Developing ethical and regulatory guidelines for research on the human microbiome and its applications: speaking to the experts and stakeholders"; "Characterizing the fecal microbiome and bacteria-derived volatile organic compounds in patients with nonalcoholic fatty liver disease (NAFLD)"; "Role of the gut microbiome in pediatric gastrointestinal illnesses"; "Elusive respiratory pathogens in the oropharyngeal flora" http://www.cihr-irsc.gc.ca/e/39951.html
Korean Microbiome Diversity using Korean Twin Cohort Project	From 2010	National Research Foundation of Korea	Seoul National University (South Korea)	To determine the microbiomes in epithelial sites of human body using Korean Twin Cohort; to investigate the relationship between body site-specific human microbiomes and diseases; to develop a Korean microbiomes diversity database

(continued)

Table 6.1 (continued)

Research program	Year	Funding source	Principal participating institutes	Program goals and website
International Human Microbiome Standards (IHMS)	2011–2015	European Commission	INRA, CEA Genoscope (France); HUVH (Spain); BCM (USA); SJTU, BGI (China); EMBL (Germany); Western College of Veterinary Medicine (Canada)	To optimize and establish standardized methods and protocols for assessing the effects of the gut microbiome on human health. http://www.microbiome-standards.org
MetaGenoPolis (MPG)	2012–2019	The French initiative "Investissements d'Avenir"	INRA, INRA-MICALIS, Université Catholique de Lyon, Cardiometabolism and Nutrition Institute (France)	To demonstrate the impact of the human gut microbiota on health and disease by making cutting-edge metagenomics technology available to the medical, academic, and industrial communities. http://www.mgps.eu

Data mainly summarized from http://www.human-microbiome.org/index.php?id=30

Table 6.2 Recent metagenomic studies showing factors affecting the human gut microbiome

Biometric factors / health conditions	Studies and major findings	References
Diet	Fecal DNA of 33 mammalian species and 18 humans were sequenced and were analyzed together with dietary information. "The adaptation of the microbiota to diet is similar across different mammalian lineages. Functional repertoires of microbiome genes, such as those encoding carbohydrate-active enzymes and proteases, can be predicted from bacterial species assemblages"	Muegge et al. (2011)
Diet	Fecal samples from 98 individuals were analyzed. Fecal communities were found clustered into the *Bacteroides* and *Prevotella* enterotypes. "Fecal communities clustered into enterotypes distinguished primarily by levels of *Bacteroides* and *Prevotella*. Enterotypes were strongly associated with long-term diets, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*). Microbiome composition changed detectably within 24 h of initiating a high-fat/low-fiber or low-fat/high-fiber diet"	Wu et al. (2011)
Diet	38 obese and 11 overweight individuals were studied. "Individuals with reduced microbial gene richness (40 %) present more pronounced dysregulated metabolism and low-grade inflammation. Dietary intervention improves low gene richness and the clinical phenotypes"	Cotillard et al. (2013)
Host genetics and family relationship	Fecal microbiota of female monozygotic and dizygotic twin pairs and their mother were compared.154 individuals were studied. "Human gut microbiome is shared among family members, but that each person's gut microbial community varies in the specific bacterial lineages present."; "A diversity of organismal assemblages can nonetheless yield a core microbiome at a functional level, and that deviations from this core are associated with different physiological states (obese compared with lean)"	Turnbaugh et al. (2009)
Host genetics and family relationship	Fecal methanogens of 40 healthy adult female monozygotic and 28 dizygotic twin pairs were analyzed. " *Methanobrevibacter smithii* pan-genome contains 987 genes conserved in all strains, and 1,860 variably represented genes. Strains from monozygotic and dizygotic twin pairs had a similar degree of shared genes and SNPs, and were significantly more similar than strains isolated from mothers or members of other families"	Hansen et al. (2011)

(continued)

Table 6.2 (continued)

Biometric factors / health conditions	Studies and major findings	References
Cultural traditions and geography	"Genes coding for these porphyranases, agarases and associated proteins have been transferred to the gut bacterium *Bacteroides plebeius* isolated from Japanese individuals."; "Seaweeds with associated marine bacteria may have been the route by which these novel carbohydrate-active enzymes were acquired in human gut bacteria, and that contact with non-sterile food may be a general factor in CAZyme diversity in human gut microbes"	Hehemann et al. (2010)
Life style and geography	Fecal microbiota of European (EU) and rural African village of Burkina Faso (BF) children were compared. BF children had significant enrichment in *Bacteroidetes* and reduction in *Firmicutes*. The genera *Prevotella* and *Xylanibacter* were abundant in BF, but not EU children. In contrast, the genera *Shigella* and *Escherichia* were significantly lower in the BF children	De Filippo et al. (2010)
Life style and geography	Feces from 531 individuals (adults and children from Venezuela, rural Malawi and US metropolitan areas) were studied. "Pronounced differences in bacterial assemblages and functional gene repertoires were noted between US residents and those in the other two countries. These distinctive features are evident in early infancy as well as adulthood"	Yatsunenko et al. (2012)
Life style and geography	Shotgun fecal metagenomes of 96 healthy Russian adults were analyzed. The sampled Russian gut microflora were similar to those found in healthy individuals. High similarities were shared among sampled rural regions, but differential abundance of metabolic pathways was shown	Tyakht et al. (2013)
Age	60 fecal samples were collected over 2.5 years from a healthy infant. "Phylogenetic diversity of the microbiome increased gradually over time"; "The earliest microbiome was enriched in genes facilitating lactate utilization, and that functional genes involved in plant polysaccharide metabolism were present before the introduction of solid food"; "Ingestion of table foods caused a sustained increase in the abundance of *Bacteroidetes*, elevated fecal short- chain fatty acid levels, enrichment of genes associated with carbohydrate utilization, vitamin biosynthesis, and xenobiotic degradation, and a more stable community composition"	Koenig et al. (2011)
Age	Fecal samples from 531 individuals (adults and children from Venezuela, rural Malawi and US metropolitan areas) were analyzed. The first 3 years of life of all 3 populations had similarity in functional maturation of gut microbiome, e.g. age-related changes in vitamin biosynthesis and metabolism genes	Yatsunenko et al. (2012)

(continued)

Table 6.2 (continued)

Biometric factors / health conditions	Studies and major findings	References
Pregnancy	Fecal compositions of 91 pregnant women and their infants were analyzed. "Gut microbiota changed dramatically from first (T1) to third (T3) trimesters, with vast expansion of diversity between mothers, an overall increase in *Proteobacteria* and *Actinobacteria*, and reduced richness."; "When transferred to germ-free mice, T3 microbiota induced greater adiposity and insulin insensitivity compared to T1…. indicate that host-microbial interactions that impact host metabolism can occur and may be beneficial in pregnancy"	Koren et al. (2012)
Obesity and metabolic syndrome	310 subjects of various body mass indices from the Old order Amish sect were studied. "22 bacterial species and 4 OTUs that were either positively or inversely correlated with metabolic syndrome traits, suggesting that certain members of the gut microbiota may play a role in these metabolic derangements"	Zupancic et al. (2012)
Type II diabetes (T2D)	345 samples of Chinese were shotgun sequenced. "Patients with T2D were characterized by a moderate degree of gut microbial dysbiosis, a decrease in the abundance of some universal butyrate-producing bacteria and an increase in various opportunistic pathogens, as well as an enrichment of other microbial functions conferring sulfate reduction and oxidative stress resistance"	Qin et al. (2012)
Type II diabetes	Fecal metagenome of 145 European women with normal, impaired or diabetic glucose control was shotgun sequenced. T2D samples had a different compositional and functional metagenome (vs. control). European and Chinese subjects had different discriminant metagenomic markers for T2D	Karlsson et al. (2013)
Obesity	Intra-individual changes of gut microbial composition before and 3 months after Roux-en-Y gastric bypass (RYGB) was studied in morbidly obese patients (body mass index (BMI) > 40 kg m^{-2}) with T2D. RYGB induced a reduction of *Firmicutes* and *Bacteroidetes* and increase in *Proteobacteria*. *Proteobacterium*, *Enterobacter* and decrease in *Faecalibacterium prausnitzii* and *Coprococcus* increased. 13 phosphotransferase system (carbohydrate metabolism) genes were significantly affected.10 species were linked to plasma total- or low-density lipoprotein cholesterol and 5 were related to triglycerides. *F. prausnitzii* directly correlated with fasting blood glucose. Correlation existed between microbiome composition, gene function and metabolic/inflammatory parameters	Graessler et al. (2012)

(continued)

Table 6.2 (continued)

Biometric factors / health conditions	Studies and major findings	References
Obesity and metabolic syndrome	The effect of infusing gut microbiota from lean donors to male recipients suffering from metabolic syndrome was tested. The insulin sensitivity and butyrate-producing gut microbiota of the recipients increased 6 weeks post treatment	Vrieze et al. (2012)
Rheumatoid arthritis (RA)	114 stool samples from RA patients and healthy controls were studied with pyrosequencing. “Presence of *Prevotella copri* as strongly correlated with disease in new-onset untreated rheumatoid arthritis patients”	Scher et al. (2013)
Kwashiorkor	Fecal metagenome was studied in 317 Malawian twin pairs during the first 3 years of life. “Combination of Malawian diet and kwashiorkor microbiome produced marked weight loss in recipient mice, accompanied by perturbations in amino acid, carbohydrate…. These findings implicate the gut microbiome as a causal factor in kwashiorkor”	Smith et al. (2013)
Gout	Fecal gut microbiota of patients with gout (n = 40) and healthy volunteers (n = 36) were compared. The genus *Shigella* was significantly increased in the gout patients, while a number of short-chain fatty acid-producing bacteria, including *Roseburia*, *Faecalibacterium* and *Subdoligranulum*, were reduced	IMAU[a]
Xenobiotic metabolism	An integrated approach combining flow cytometry, pyrosequencing and metatranscriptomic analysis showed that xenobiotic exposure significantly activated a specific set of gut microbes, mainly *Firmicutes*. Multiple responsive genes relating to antibiotic resistance, drug metabolism and stress responsive pathways were also identified from different bacterial phyla	Maurice et al. (2013)
Nonalcoholic fatty liver disease (NAFLD)	The gut metagenome (pyrosequencing) and metabolome (gas chromatography) of 60 NAFLD and healthy subjects were studied. An increase in *Lactobacillus* and some *Firmicutes* (*Lachnospiraceae*; genera, *Dorea*, *Robinsoniella*, and *Roseburia*) was observed in almost all NAFLD fecal metagenome. NAFLD fecal metabolome had significant increase in fecal ester compounds	Raman et al. (2013)
Antibiotic usage	252 fecal metagenomes were screened for 68 classes/subclasses of antibiotic genes. The most abundant resistance genes were those having a long history of use, especially in animals. Samples from Spain, Italy and France had more antibiotic resistance genes than those from US, Denmark or Japan. Moreover, antibiotic resistance determinants persisted in human gut flora for >1 year	Forslund et al. (2013)

(continued)

Table 6.2 (continued)

Biometric factors / health conditions	Studies and major findings	References
Antibiotics treatment	The long- and short-term effects of clarithromycin and metronidazole treatment on fecal microbiota were monitored over a 4-year period. "Dramatic shifts were observed 1 week after antibiotic treatment with reduced bacterial diversity in all treated subjects"; "The microbiota of the different subjects responded uniquely to the antibiotic treatment some general trends could be observed; such as a dramatic decline in *Actinobacteria* in both throat and feces immediately after treatment"; "The microbiota remained perturbed in some cases for up to 4 years post treatment"	Jakobsson et al. (2010)
Antibiotics treatment	Fecal samples of 3 subjects (52–56 samples per subject) were analyzed over 10 months spanning 2 antibiotic ciprofloxacin courses. "Community changes after ciprofloxacin varied among subjects and between the two courses within subjects. In all subjects, the composition of the gut microbiota stabilized by the end of the experiment but was altered from its initial state"	Dethlefsen and Relman (2011)
Clostridium difficile colitis	Fecal microbiota of patients with antibiotic-associated diarrhea due to *Clostridium difficile* (CDAD) was studied. Bacterial compositions in CDAD recurrent patients were highly variable with strongly reduced microbial diversity, compared to control subjects or patients at initial phase of disease	Chang et al. (2008)
Clostridium difficile colitis	The effect of fecal transplantation in CDAD patients was monitored. By day 14 of fecal transplantation of fecal bacteria from healthy individuals, the gut microflora of recipients were very similar to that of the donors, dominated with *Bacteroides* and an undefined butyrate producer. The treatment also resulted in symptom relief. Thus, fecal transplantation was able to restore the structure and function of originally deficient gut microbes in CDAD patients	Khoruts et al. (2010)
Other inflammatory bowel diseases	Mucosal samples of 40 twin pairs, with concordant/discordant for Crohn's disease (CD)/ulcerative colitis (UC); patients were in remission or with active disease. Gut microbiome of CD, but not UC, differed from those of healthy individuals. Gut microbial profiles of ileum and colon CD patients were disease group-specific. Ileal CD patients had reduced *Faecalibacterium* and *Roseburia*, and increased *Enterobacteriaceae* and *Ruminococcus gnavus*	Willing et al. (2010)

[a] Key Laboratory of Dairy Biotechnology and Engineering (Ministry of Education, China), Inner Mongolia Agricultural University

Functional metagenomic sequencing results of a healthy western cohort published by the HMP Consortium covered an estimate of 81–90 % of the genera, enzyme families and community configurations (Human Microbiome Project Consortium 2012). A conserved set of metabolic pathways could be identified though variation existed between individuals, and that ethnic/racial background seemed to bear strong link with the gut metagenome. Moreover, the data revealed a general enrichment of genes relating to metabolism (particularly, pathways of carbohydrate/energy metabolism, short-chain fatty acid generation, amino acid metabolism, secondary metabolite biosynthesis, and cofactors/vitamin metabolism), as compared to the human genome based on Clusters of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) categorizations (Candela et al. 2010).

The enrichment of genes involving carbohydrate metabolism is further demonstrated by Turnbaugh et al. (2009) that found at least 156 genes belonging the carbohydrate-active enzyme (CAZymes) families (including 77 glycoside hydrolase, 21 carbohydrate-binding module, 35 glycosyltransferase, 12 polysaccharide lyase and 11 carbohydrate-esterase family genes) within each of the 18 analyzed gut microbiomes. There was a higher average of CAZymes in the gut microbiome than the most abundant KEGG pathways (2.6 % vs. 1.2 %). The sharing of a conserved functional gene set and a high diversity of gut microbial-originated CAZymes between individuals are also supported by the results from a recent functional metagenomic screening for prebiotic-hydrolyzing enzymes targeting inulin, fructo-oligosaccharides, xylo-oligosaccharides, galacto-oligosaccharides, and lactulose. Among the identified proteins, 20 of them were at least present in 100 subjects regardless of their age or geographical origin (Cecchini et al. 2013). These CAZymes encoded by the human gut microbial counterpart might enable the utilization of the normally “indigestible” glycans of plant origin (e.g., xylan, pectin, cellulose).

6.1.4 Toward a Core Normal Gut Microbiota?

Multiple efforts have tried to define a set of “core” normal gut microbiota shared between every human. Based on NGS surveys, the gut microbiome of each individual is made up to 6–10 gut bacterial phyla with the dominating phyla of *Bacteroidetes* and *Firmicutes*, as well as the less prevalent ones including *Acidobacteria*, *Actinobacteria*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria*, candidate division TM7, *Verrucomicrobia*, and *Deinococcus-Thermus* (Marchesi 2010). However, at a lower taxonomical level, 70 % out of the 16,000 described gut phylotypes were individual-specific with no phylotype existed at over 0.5 % in all subjects (Maccaferri et al. 2011).

A large-scale nationwide survey was performed by the Key Laboratory of Dairy Biotechnology and Engineering (Ministry of Education, P. R. China) to characterize the phylogenetic metagenome of 314 Chinese individuals from 7 ethnic groups living in nine different provinces, namely, Han (the major Chinese ethnicity group),

Bai, Zhuang, Uyghur, Kazakh, Tibetan, and Mongol. The different ethnic groups formed distinct clusters upon canonical analysis of unweighted UniFrac principal coordinates, whereas the lifestyles of the subjects (urban or rural) did not seem to be as influential as the subjects' ethnic origin. Moreover, fecal specimens of all the subjects shared nine core bacterial genera, which accounted for a total of 47.63 % of analyzed sequences in the study. These genera included *Phascolarctobacterium* (13.16 %), *Roseburia* (6.7 %), *Bacteroides* (5.54 %), *Blautia* (4.78 %), *Faecalibacterium* (4.63 %), *Clostridium* (4.58 %), *Subdoligranulum* (3.42 %), *Ruminococcus* (2.78 %) and *Coprococcus* (2.04 %). However, at the operational taxonomic unit (OTU) level, most OTUs were shared by a limited number of subjects and none of them was found in all individuals (unpublished data).

Arumugam et al. (2011) combining data from fecal metagenomes of six countries and proposed a three enterotype model, dominated by one of the genera *Bacteroides*, *Prevotella* or *Ruminococcus*. The study also concluded that irrespective of individual host properties such as body mass index, age, or gender, fecal metagenomes fell into one of these enterotypes. This enterotype model remains controversial and is viewed as oversimplified, especially if a larger sample size or different geographical original/background subjects would be considered (Yatsunenko et al. 2012; Jeffery et al. 2012). On the other hand, Koren et al. (2013) combined the HMP 16S rRNA gene sequence data with other published data, and assessed the enterotype pattern across different body sites. They found smooth abundance gradients of key genera for most sample sets and body sites rather than an enterotype clustering pattern. Moreover, they concluded that the methodology employed to analyze the data influenced the likelihood in identifying and classifying enterotype; therefore, multiple approaches should be used to allow an accurate presentation and interpretation.

With the availability of enormous amounts of data on the gut microbial reference genomes and gene catalogs, theoretically, it should be easy to define a "core" normal gut microbiota shared by all or most healthy individuals. However, due to the high individual-specificity, it does not seem to be the case, at least at the species or OTU level (Qin et al. 2010; Human Microbiome Project Consortium 2012). Thus, perhaps a potentially more meaningful question to ask is how different physiological status is related to the gut microbiome composition at personalized level. Moreover, instead of a core gut microbiota, it seems to be more feasible to define a core set of microbial functional metabolic pathways, which may also serve as valuable health-status biomarkers.

6.1.5 An Integrated 'Omics' Approach to Study Gut Microbiome

Apart from applying metagenomics into the microbial phylogenetic description of an ecological site like the gut environment, functional roles at community level can be assigned and studied by shotgun sequencing of the whole genome collected

at the sites of interest. However, this functional metagenomics approach does not provide clear information on the active portion of the gut microbiome or metagenome, and even fewer reports focused on analyzing the inter-individual variation of the active portion of the gut metagenome. As a matter of fact, the active microbial members in the gut do not always correlate with the total bacterial amount (Peris-Bondia et al. 2011). Smith et al., by an integrated approach utilizing flow cytometry, pyrosequencing, and metatranscriptomics analyzed the gut metagenome upon short-term exposure to a variety of xenobiotics. Only a distinctive and inter-individual-specific set of microbes, mainly from *Firmicutes*, seemed to be active. An array of antibiotic resistance, drug metabolism, and stress responsive pathways was differentially expressed, and the involved genes spanned across different bacterial phyla (Maurice et al. 2013).

By using a metatranscriptomic approach (Gosalbes et al. 2011), the *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Prevotellaceae*, and *Rickenellaceae* families were found to be the predominant active members in the gut microbiome. The gut metatranscriptome mainly serves for carbohydrate and energy metabolism, and cellular component synthesis rather than other housekeeping functions. A metaproteomic study by shotgun mass spectrometry was performed to characterize the protein composition in human fecal samples (Verberkmoes et al. 2009). The generated metaproteomes was found to have a higher level of proteins for translation, energy production, and carbohydrate metabolism, as compared to the fecal metagenome.

Metabolomics is another approach which has been used to study the activities of the gut microbiome. A recent metabolomic-based study performed in germ-free mice elegantly showed the critical role of dietary choline and gut microflora in trimethylamine N-oxide production, which in turn led to the upregulation of multiple macrophage scavenger receptors and, subsequently, the enhancement of atherosclerosis (Wang et al. 2011). The metabolomic approach has also been applied to study chronic gut inflammatory disorders like ulcerative colitis (UC) and IBS. Nuclear magnetic resonance (NMR) spectroscopy together with PCR-DGGE was used to compare the aqueous fecal extract compositions between normal individuals, and patients suffering from UC or IBS (Le Gall et al. 2011). Taurine and cadaverine levels increased in UC patients, whereas a higher amount of bile acid and a reduction of branched chain fatty acids were observed in the case of IBS. Cluster analysis of the DGGE profile and NMR spectrum data partly distinguished the three sample groups and correlated the gut microbiota composition and metabolite profile.

Raman et al. (2013) combined the metagenomic and metabolomic approaches to study the etiology of nonalcoholic fatty liver disease (NAFLD). In the study, the gut metagenome and metabolome of 60 subjects (30 NAFLD and 30 healthy controls) were analyzed with pyrosequencing and gas chromatography, respectively. An increase in *Lactobacillus* and several *Firmicutes* genera (*Lachnospiraceae*, *Dorea*, *Robinsoniella*, and *Roseburia*) were noted in the fecal metagenome of all but one NAFLD patients. Such variation was accompanied by a different volatile organic compound profile with significant increase in fecal ester

compounds, suggesting that the gut microbiota played a role in altering the GIT microenvironment and physiology.

All of the aforementioned "omics" approaches are powerful methods to decipher scientific questions. However, each has its own strength and weakness. It is anticipated that a "multi-omics" approach will be increasingly implemented to broaden our knowledge in defining the activities of the gut microbiome, and molecular events at protein and metabolite levels.

6.1.6 The Underexplored: Gut Viruses, Fungi and Archaea

The gut microbiota is composed of prokaryotes, eukaryotes, and viruses. Among them, the bacterial members are focused in most metagenomic studies and have been fully described. Other members, including the groups of viruses, fungi, and archaea, are often neglected or understudied.

6.1.6.1 The Gut Viruses

Accumulating evidence shows that the gut virome may influence human health. Recently, the roles of common viral infections in elevating the risk for developing complex diseases, such as type-1 diabetes, inflammatory bowel disease, and asthma, have been proposed (Foxman and Iwasaki 2011). The presence of certain gut resident viruses, e.g., polyomaviruses, is associated with diseases (Siebrasse et al. 2012). Moreover, gut resident viruses may serve as flexible gene pool and gut mobile metagenome (e.g., antibiotic resistance and virulence genes), which coevolve with the host and the gut microbiota (Ogilvie et al. 2012, 2013). However, current metagenomics studies on the gut microbiota mainly focused on the bacterial members, and largely neglected the gut virome. Therefore, the viral diversity present in the human body sites and their biology are not adequately understood.

An abundant and diverse community of both DNA and RNA viruses exist in the adult GIT environment (up to 17 % of fecal-extracted microbial DNA) (Ogilvie et al. 2013). Among them, the DNA viruses are mostly phages, whereas most of the RNA viruses are of plant origin (Breitbart et al. 2008). More recent metagenomic studies have reinforced the fact that the fecal/gut virome was dominated with prokaryotic phages (Wylie et al. 2012), and potentially the bacteriophage particles were present in higher number in the GIT than bacterial cells (Reyes et al. 2010). Furthermore, mimiviruses and marseilleviruses, both belong to the new virus order of *Megavirales*, were reported in human gut (Colson et al. 2013). In addition, these studies revealed a high inter-individual variation in stool bacteriophage communities and an age-related stratification. Around 10–984 (in 12 adult subjects) and 19–785 viral genotypes (in 16 adult subjects) per fecal sample were reported in two separate publications (Reyes et al. 2010; Minot et al. 2011), contrasting to the extremely low viral diversity detected in infants (1930 vs. 8 in adults vs. infants reported in Breibart et al. (2008)). Another recent metagenomic study on the fecal

samples of two extremely low birth weight neonates (within 1 month old) described the presence of a small percentage of virus-originated sequences corresponding to the single-stranded DNA viruses (bacteriophages S13, phiX174, alpha 3), double-stranded phages (*Staphylococcus* Phage K, a *Caudovirales* species with a *Staphylococcus* host) and a rare human adenovirus C (LaTuga et al. 2011). These data together suggest that the gut virome diversity evolves and develops through the different life stage of the subjects. Moreover, in another study, the gut virome structure was found to be alterable by dietary change (Minot et al. 2011).

The high inter-individual variation of gut virome was further demonstrated in Reyes et al. (2010) that analyzed the fecal samples of co-twins and their mother. Their results suggested that, contrasting to the gut bacterial communities, the gut virome composition was independent from genetic relationship. A remarkably low intra-individual diversity of >95 % virotypes retained over the monitored period of over 1 year, and the virome population was dominated by several highly genetically stable temperate phages. However, the observation of the high inter-individual gut virome variation was challenged by two recent studies, both bioinformatically searched for phage-specific signatures from available metagenomes. Stern et al. (2012) used the clustered regularly interspaced short palindromic repeats loci together with their associated *cas* genes (defense system against propagation of phages and plasmids) to identify the phage composition based on the MetaHIT data (Stern et al. 2012), whereas Ogilvie et al. attempted to describe the '*Bacteroidales*-like' phages based on tetranucleotide usage profiles (Ogilvie et al. 2013). Both studies concluded that a common reservoir of phages was broadly shared between individuals, although a subset of lysogenic prophage might be preferentially associated with specific hosts as indicated by their high abundance (Stern et al. 2012). Yet, the contradictory view seems to be resolvable by the model proposed by Minot et al. (2013) that monitored the fecal viral community of one subject for over 2.5 years, and 80 % of the viruses persisted during the studied period. However, the mutation rate varied between different viral groups. Temperate phages had comparatively lower mutation rates, whereas *Microviridae* showed high substitution rates which were enough to form new viral species over the 2.5-year period. Hence, the high inter-individual difference may be explained by the rapid evolution of some of the members, whereas everybody does share a pool of relatively stable gut viral population.

Even though more recent studies have focused on the human gut virome structure and how it is modulated by genetic and environmental factors, the current understanding in these areas remains limited. Moreover, whether the human gut virome is highly personalized or similar between individuals, and to what extent and its significance, is still to be further explored.

6.1.6.2 The Gut Fungi

Fungi are the dominant eukaryotic gut microbiome members in healthy subjects (Nam et al. 2008; Hamad et al. 2012). Only very few comprehensive surveys

focused on the human fungal gut microbiome composition and, therefore, the current understanding on their roles in human gut health is insufficient (see Table 6.3 for a review on some of the surveys performed on the gut fungi). Moreover, most studies so far relied on the more traditional low-throughput methods like laboratory cultivation methods, fungal ITS/18S rDNA-based polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) fingerprinting and clone library, in situ hybridization techniques (Nam et al. 2008; Ott et al. 2008; Scanlan and Marchesi 2008). Only a low diversity of the fecal/gut fungal community was detected based on these methods (6.2 ± 2.4 in normal individual) (Ott et al. 2008). Hamad et al. (2012) applied PCR with different fungal target genes in combination with clone library, DNA sequencing, traditional culture methods, and MALDI-TOF, and identified 17 different species.

Metagenomic surveys based on high throughput sequencing of 18S rDNA of fungal populations of the human feces samples are limited to few publications. Hoffmann et al. (2013) reported a diverse fungal community of 66 genera belonging to the phyla *Ascomycota* and *Basidiomycota*. The two phyla were negatively correlated to each other in most of the samples. *Saccharomyces*, *Candida* and *Cladosporium* were the three most dominant genera (present in 89, 57 and 42 % of the samples, respectively), and the gut resident fungal composition was related to recent dietary intake. For instance, the genus *Candida* was correlated positively with carbohydrates and negatively with total saturated fats, whereas *Aspergillus* was negatively correlated with short-chain fatty acids. Surprisingly, no observable trend was shown in *Saccharomyces*. In the feces of some of the extremely low birth weight infants, the gut fungiome was dominated with *Candida* and *Clavispora* species along with other environmental molds (*Cladosporium*, *Cryptococcus* and *Saccharomyces*) (LaTuga et al. 2011).

Some fungi, for example, *Saccharomyces boulardii* is regarded as a probiotic gut microbiota, and showed encouraging protective effects in human clinical trials for diarrhea and other gastrointestinal diseases (Parfrey et al. 2011; Kelesidis and Pothoulakis 2012). In preterm infants, it improved feeding tolerance, reduced the risk of clinical sepsis and invasive fungal colonization and infection (Demirel et al. 2013a, b). Another fungus, *Candida albicans*, is detected in the feces of some normal individuals. It usually does not draw clinical attention except in the pathogenesis caused by an overgrowth. This fact may implicate that the specific fungus plays an opportunistic role, and possibly, acts as a factor that jeopardizes the gut microbiome balance and leads to gut dysbiosis. Some of the triggers, which may disrupt such balance, include the application of antibiotics, corticosteroids, and immunosuppressive drugs (Schulze and Sonnenborn 2009). Moreover, the fungal diversity in fecal samples of Crohn's disease patients was found to be deviating from normal individuals, and the exact role of alteration of the gut fungal populations and how such change interacts with other resident microflora during the disease development remain to be determined (Ott et al. 2008).

The composition and function of the human gut fungiome need to be further dissected by the state-of-art NGS technology more thoroughly and in-depth. To the best of our knowledge, so far all the reported gut fungi belong to the phyla

Table 6.3 Some recent surveys on the gut fungi

Study approaches	Phyla	Reported fungi	Sample types	References
PCR-DGGE fingerprinting	*Ascomycota*	*Saccharomyces servazzii, Saccharomyces cerevisiae, Candida edaphicus, Candida vinaria*	Stool	Nam et al. (2008)
18S rDNA PCR DGGE, clone libraries, sequencing, and in situ hybridization	*Ascomycota Basidiomycota*	*Candida* (*albicans, glabrata*), *Penicillium* (*italicum, glabrum, sacculum, verruculosum, chrysogenum*), *Saccharomyces* (*cerevisiae, cariocanus, bayanus*), uncultured *ascomycetes,* 1 uncultured *basidiomycetes*	Biopsy tissues	Ott et al. (2008)
ITS/18S rDNA PCR DGGE, clone libraries, sequencing, culture	*Ascomycota Basidiomycota*	*Gloeotinia temulenta* (*Paecilomyces fumosoroseus*), *Cephalosporium sp, Saccharomyces cerevisiae, Aspergillus versicolor, Galactomyces geotrichum, Galactomyces sp BPY-54, Penicillium roqueforti, Candida albicans, Acremonium sp, Psathyrella candolleana* (the only *Basidiomycota* member)	Stool	Scanlan et al. (2008)
PCR of different fungal target genes, clone library, culture, MALDI-TOF	*Ascomycota*	*Galactomyces geotrichum, Saccharomyces* (*cerevisiae, pretoriensis, telluris*), *Kluyveromyces hubeiensis, Geotrichum candidum, Candida rugosa*	Stool sample of a 16-year-old Senegalese man	Hamad et al. (2012)
PCR of different fungal target genes, clone library, culture, MALDI-TOF	*Basidiomycota*	*Asterophora parasitica, Sterigmatomyce elviae, Bjerkandera adusta, Phanerochaete stereoides, Trichosporon* (*caseorum, cutaneum, asahii*), *Malassezia* (*globosa, restricta, pachydermatis*)	Stool sample of a 16-year-old Senegalese man	Hamad et al. (2012)
NGS	*Ascomycota*	*Candida* (*albicans, glabrata, quercitrusa, diddensiae, parapsilosis, tropicalis*), *Cladosporium* (*cladosporioides, sphaerospermum, tenuissimum*), *Clavispora, Saccharomyces cerevisiae*	Stool samples of extremely low birth weight infants	LaTuga et al. (2011)
NGS	*Basidiomycota*	*Cryptococcus* (*albidosimilas, podzolicus*)	Stool samples of extremely low birth weight infants	LaTuga et al. (2011)
NGS	*Ascomycota*	*Alternaria, Cladosporium, Aspergillus, Candida, Debaryomyces, Meyerozyma, Pichia, Saccharomyces, Fusarium*	Stool	Hoffmann et al. (2013)
NGS	*Basidiomycota*	*Rhodotorula, Cryptococcus, Cystofilobasidium*	Stool	Hoffmann et al. (2013)

Ascomycota and *Basidiomycota*. The discovery of novel species and a wider gut fungal diversity is anticipated with a more extensive sequencing and an increase in sample size and variety of geographic/ethnic origins.

6.1.6.3 The Gut Archaea

Archaea were first discovered in extreme natural environments (Dridi et al. 2011). Although the appearance and size of archaea and bacteria are alike, they are phylogenetically separated based on 16S rDNA clustering analysis. Archaea are closer to the eukaryotes both in terms of their gene regulation and metabolic pathways. Only very few members of this group are cultivable under the laboratory condition. The difficulty in laboratory cultivation and the plausible presence of only low number of some of the archaeal members have largely limited the study of gut-associated archaea (see Table 6.4 for some of the reported gut-associated archaea).

In the 1980s, the first two archaea, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, were isolated and cultivated from human feces samples under strict anaerobic conditions (Miller and Wolin 1985; Miller et al. 1982). The presence of these species in the human gut was further confirmed by a survey of the methanogens in the fecal samples of 20 subjects (Brusa et al. 1993). Early metagenomics studies by Eckburg et al. (2005) and Gill et al. (2006) confirmed the high prevalence of *Methanobrevibacter smithii* and suggested a low diversity of archaea in the human gut. In both studies, all the archaeal DNA sequences were found to be *Methanobrevibacter smithii*. Up to one-tenth of all colon anaerobes in healthy adults were comprised of methanogenic archaea (Eckburg et al. 2005). In a more recent study, by 16S rDNA sequence analysis, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* were detectable in 99.2 and 32.6 % of stool samples, respectively (Dridi et al. 2012a).

Not until lately, two other novel *Thermoplasmatales*-related methanogenic archaea, *Methanomassiliicoccus luminyensis* and *Candidatus Methanomethylophilus alvus*, were isolated (Borrel et al. 2012; Dridi et al. 2012b). *Methanomassiliicoccus luminyensis* was detectable in 4 % of the human fecal samples collected in France. This species was distinct from other human associated archaea in the way that it could use methanol in the presence of hydrogen. Moreover, *Methanomassiliicoccus luminyensis*, but not *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, was found to be significantly increased with age (Dridi et al. 2012a, b). *Candidatus Methanomethylophilus alvus* was isolated from enrichment culture of feces of the elderly, and it is distantly related to *Methanomassiliicoccus luminyensis* with 87 % 16S rRNA gene sequence identity (Borrel et al. 2012).

Yet, later metagenomic analysis has presented a different picture on the gut archaeal composition with a wider diversity, including both methanogenic and nonmethanogenic members. Hoffmann et al. (2013) reported the detection of five archaeal genera from 96 fecal samples, and 44 of them contained more than one species. *Methanobrevibacter* and *Nitrososphaera* species were positive in 31.3 and

Table 6.4 Some of the reported fecal archaea

Study approaches	Phyla	Reported archaea	References
Metagenomics	*Euryarchaeota*	*Methanobrevibacter smithii*	Eckburg et al. (2005)
Metagenomics	*Euryarchaeota*	*Methanobrevibacter smithii*	Gill et al. (2006)
Metagenomics	*Euryarchaeota*	*Methanobacteriales* and undefined archaea	Zhang et al. (2009)
Metagenomics	*Euryarchaeota*	*Methanobrevibacter*, *Methanosphaera*, *Thermogymnomonas*, *Thermoplasma*	Hoffmann et al. (2013)
Metagenomics	*Thaumarchaeota*	*Nitrososphaera*	Hoffmann et al. (2013)
Laboratory cultivation	*Euryarchaeota*	*Methanosphaera stadtmanae*	Miller and Wolin (1985)
Laboratory cultivation	*Euryarchaeota*	*Methanomassiliicoccus luminyensis*	Dridi et al. (2012a, b)
Enrichment culture of fecal samples from elderly subjects	*Euryarchaeota*	*Candidatus Methanomethylophilus alvus*	Borrel et al. (2012)
PCR 16S rRNA	*Crenarchaeota*	*Sulfolobales* and undefined species	Rieu-Lesme et al. (2005)
PCR 16S rRNA	*Euryarchaeota*	*Methanobrevibacter smithii*, *Methanosphaera stadtmanae*, *Halorubrum koreense* strain B6, *Halococcus morrhuae* NRC 16008	Nam et al. (2008)
PCR 16S rRNA	*Euryarchaeota*	*Halobacteriaceae*	Oxley et al. (2010)
PCR *mcrA*	*Euryarchaeota*	*Methanosarcinales* (DC UC-6, RFLP type D)	Scanlan et al. (2008)

16.7 % of samples, respectively. These two genera were mutually exclusive except for six samples. Furthermore, the archaeal subpopulations were found to be associated with different dietary intake. *Methanobrevibacter*-positive samples were correlated with a higher long- or short-term carbohydrate intake, whereas *Nitrososphaera*-positive samples were correlated with a high long-term intake of vegetable or polyunsaturated fat. Lower prevalence archaeal members reported in the same study included *Methanosphaera*, *Thermogymnomonas* and *Thermoplasma*. More human GIT-associated archaea members, including *Methanosarcina*, *Crenarchaeota* and halophilic archaea, were revealed by other molecular-based studies (Dridi et al. 2011). Most likely, owing to their low abundance, these genera have not been detectable by the metagenomic approach. Surveys based on these improved molecular detection together with NGS methods reveal that archaeal members are more prevalent and diversified than originally thought.

The major gut archaeal species, *Methanobrevibacter smithii*, is ubiquitous in and well-adapted to the human gut by having wide polysaccharide fermentation

capacity (Samuel et al. 2007; Dridi et al. 2009). The resultant fermentation products, e.g., short-chain fatty acids (SCFA), may subsequently regulate the host gut metabolism and physiology. Interestingly, obese individuals had elevated levels in both fecal archaeal abundance and short-chain fatty acid content compared to normal weight or postgastric-bypass subjects (Zhang et al. 2009; Patil et al. 2012). Thus, it was proposed that gut archaeal population might play key roles in enhancing energy uptake in obese intestine by interspecies H_2 transfer (Zhang et al. 2009). On the other hand, contrary data from Million et al. suggested that human obesity was related to the reduction of gut *M. smithii* (Million et al. 2012). Nonetheless, an increased proportion of fecal archaea may also be associated with chronic gut disorders like Crohn's disease, IBS, and colorectal cancer (Nakamura et al. 2010; Roccarina et al. 2010).

All the four human gut cultivable archaeal members are strict anaerobe and methanogenic. The methanogenesis process certainly beneficially prevents from an excessive accumulation of acids and other reaction end-products in the gut. However, their exact roles in human health, in particular energy uptake, obesity and chronic gut disorders, are to be further investigated.

6.1.7 Limitations and Challenges

With the advance of NGS technology and the wide availability of bioinformatic tools, rapid breakthrough on the knowledge and understanding of the gut microbiome has been achieved within the past decade. However, certain challenges and limitations remain in the field.

First, up to now there are enormous amounts of data generated from different studies including subjects from different geographic regions and ethnicities. Meanwhile, there are huge variation and contradictions in the reported data in the field. Most likely, the high inter-individual variation contributes to a large extent to the observed differences, and that the gut microbiome is strongly associated with the host genetic background and ethnicity (Human Microbiome Project Consortium 2012). However, several other technical aspects may contribute together to the observed discrepancies. Hugon et al. compared microbiota in 16 human fecal samples using Gram staining, flow cytometry, transmission electron microscopy, qPCR and pyrosequencing of the 16S rDNA amplicon of V6 region, and concluded that the Gram-negative-like populations in the human gut microbiota were largely underestimated (Hugon et al. 2013). Such results have demonstrated the bias of relying on one single technique, e.g., pyrosequencing, to assess complex communities like the gut microbiota, and the significance of using multiple methods to gain a more complete and realistic picture.

Other technical aspects and data handling in the metagenomic approach may also be partly responsible for the high variability. For example, the choice of primers used to amplify the 16S rDNA in phylogenetic metagenomic studies may produce biased results. The relative abundances of phyla were found differing

significantly by designing primers based on different hypervariable regions. *Bacteroidetes* was found to be the prevailing phylum using 16S rDNA v4/v5 region primers, whereas *Firmicutes* was dominating if 16S rDNA v3/v4 primers were used on the same samples (Claesson et al. 2010). Data from Andersson et al. largely deviated from other pyrosequencing studies with a drastic decrease in *Bacteroidetes* (only 2.5 %) and an increase in *Actinobacteria* (12.5 %), which was probably due to the primer choice as pointed out by the authors (Andersson et al. 2008). Another possible technical reason is the laboratory procedures like DNA storage and extraction. Various studies showed that changes in sample storage conditions or DNA extraction methods might produce different outcomes in metagenomic experiments and potentially lead to errors (Cardona et al. 2012; Maukonen et al. 2012). Owing to the large intrinsic intra-individual variation, it is, therefore, very hard to judge whether any difference between studies is truly attributed to the intrinsic sample difference or other technical reasons. Without a set of well-defined guidelines and uniformed protocols, it will be problematic to compare and interpret data generated between different studies/laboratories, thus limit the applicability of these results in a global sense.

Second, one of the major goals of the HMP is to create an extensive gene-catalog and reference genome database of common human gut microbes. Up to now, over 1000 reference genomes have already been sequenced. However, a recent analysis, by comparing the HMP's 16S dataset with several reference 16S collections, revealed that many gut microbes are still underrepresented, in particular the rare or low-abundant lineages (Fodor et al. 2012). Similarly, many of these "most wanted" phylotypes are not held in currently available culture collections, implying that our current reference genome database is still far from complete. Therefore, there is an urgent need to improve the sensitivity of the current metagenomic sequencing techniques for the detection of rare/low-abundant members, as well as to develop novel culture- and/or single-cell-based methods in isolating the minority taxa.

Third, there is yet a large knowledge gap in defining the activities and functions between the microbial metagenome and the individual gut residents, and the mechanisms of microbial-host interaction remain largely unknown. Traditionally, the study of the gut microbiota and their functions relied heavily on laboratory cultivation techniques. However, it is estimated that over 80 % of the gut microbes are unculturable (Maccaferri et al. 2011). Recently, the idea of "culturomics" was developed based on systematically culturing microbes under different conditions, and followed by MALDI-TOF identification. Such method complemented the metagenomic approach, and led to the isolation of 31 new bacteria (Lagier et al. 2012). In Pfleiderer et al. (2013), 11 novel bacterial species were identified by a joint culturomics and pyrosequencing strategy from a single anorexia nervosa stool sample. In this way, both the cultivation sensitivity and the efficiency of identification of yet unculturable species are greately improved, so that further studies of microbial function and host interaction are enabled.

6.2 Lactic Acid Bacteria in the Human Gastrointestinal Tract

6.2.1 The Human GIT and Its Functions

The human GIT is a long ($\sim$8.3 m) tubular structure of mucous membrane and muscle extending from mouth to anus (Prakash and Malgorzata Urbanska 2008). The human GIT mucosal surface (about 200–300 m^2) hosts a diverse bacterial population (10^{13-14} bacteria of around 400 different phylotypes) (Hao and Lee 2004). The GIT can be viewed as different compartments, including the mouth cavity, esophagus, stomach, small and large intestine, which are tailored for their designated functions. The principal function of the human GIT is for the digestion of feedstuffs and absorption of nutrients. Apart from this, it plays crucial roles in osmoregulation, endocrine regulation of digestion and host metabolism, immunity and defense against potential pathogens, as well as detoxification and elimination of host generated and environmentally acquired toxic chemicals. These functions can only be properly performed by the interaction and coordination of the various compartments and components of the GIT (Fig. 6.2).

Each of the GIT compartments is unique in its physical, chemical, and biological features, and forms habitats of distinct ecology. The abiotic and biotic factors are interacting together to shape the structure and composition of the human GIT microflora (Table 6.5). Several abiotic factors, namely pH, peristalsis, and redox potential, are of particular importance in determining the prevalence of bacteria in a specific gut micro-habitat. Among the different GIT compartments, the stomach and the proximal small intestine usually contain relatively low bacterial mass because of the high acidity and swift peristaltic action. The colon is characteristic of its anoxia and low redox potential. The colon lumen is normally packed with a much higher number and diversity of bacteria, over 90 % of which are anaerobes (Roediger 1980). The anaerobic colonic environment enhances the fermentation of carbohydrate to form SCFA, which in turn provides energy both for the enterocytes and some of the beneficial bacteria like *Lactobacillus*. The pattern of intestinal peristalsis is stimulated by SCFA and hence the gut resident bacteria (Grider and Piland 2007).

Apart from the abiotic factors, the prevalence and structure of the GIT microbiota are influenced by a number of biotic factors like the host features (e.g., genetics, physiology, immunity, age) and microbial capacity and interaction (e.g., cell adhesion ability, mutualism/antagonism/competition, mucin secretion, nutrient uptake/utilization). The GIT microbiota can be autochthonous (indigenous) or allochthonous (transient) in nature. Autochthonous floras are the true residents, which can colonize particular habitats and become the stable community. In contrast, allochthonous microbes are only “in transit,” most likely sourcing from ingested food and water (Ley et al. 2006). The allochthonous flora may have a lower capacity to stably colonize in the human GIT environment unless the normal resident microflora is perturbed. The communities of gut microbiota together participate in the functioning and homeostasis of the human GIT.

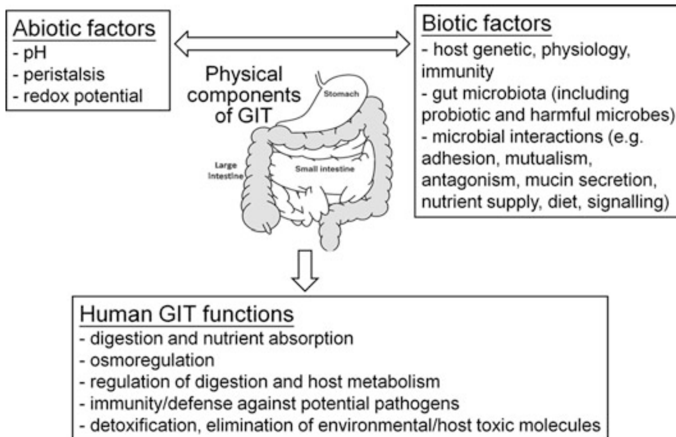


Fig. 6.2 The environment of human GIT and its functions

6.2.2 Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) is a diverse group of Gram-positive facultative anaerobic bacteria with a fermentative metabolism (Kandler 1983). This group of bacteria is associated with the human GIT, and confers functional properties to the host. Therefore, they hold strong link with human health. Moreover, they are useful fermentation starter cultures and are commonly found in food. Thus, they are good choices for commercial product application and are often marketed for disease prevention and treatment. The majority of the currently available probiotic strains used in food and probiotic-based supplements are LAB, in particular the genera *Lactobacillus* and *Bifidobacterium* (Foligné et al. 2013).

LAB belong to the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales*. This group of organisms has relatively small genome size, and, therefore, they may have restricted metabolic functions and are heavily relying on a direct environmental nutrient supply (Makarova and Koonin 2006). Owing to such constraint, their natural habitats are often limited to plants (cabbage, corn, barley, mashes, kale, and silage), meat, dairy, and the gut of animals/human (Carr et al. 2002). The diverse group of LAB is grouped into six families (*Aerococcaceae*, *Carnobacteriacea*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae*) and around 20 genera (common genera include the *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella*, see Table 6.6). The genus *Bifidobacterium* (phylum *Actinobacteria*, class *Actinobacteria*, order *Bifidobacteriales*) is phylogenetically unrelated to the aforementioned LAB members, but is universally regarded as LAB due to its metabolic commonality in lactic acid production.

Table 6.5 Microbial load and diversity in the GIT compartments

GIT compartment	pH	Microbial load (cells/mL)	Autochthonous microbes	Allochthonous microbes
Oral cavity	6.5–7.5	10^{8-9}	*Gemella* (e.g. *G. haemolysans*), *Granulicatella*, ***Streptococcus*** (e.g. ***S. mitis***), *Veillonella*, *Prevotella*, *Porphyromonas*, *Rothia*, *Neisseria*, *Fusobacterium*, ***Lactobacillus***	Dominated by autochthonous microbes
Esophagus	4–6	10^{2-3}	Prevalent taxa: ***Streptococcus***, *Neisseria*, *Veillonella* Less prevalent taxa: *Fusobacteria*, *Bacteroides*, ***Lactobacillus***, *Staphylococcus*, yeasts, enterobacteria	
Stomach	1–5	10^{2-3}	*Helicobacter pylori*	*Gemella* (e.g. *G. haemolysans*), *Granulicatella*, ***Streptococcus*** (e.g. ***S. mitis***), *Veillonella*, *Prevotella*, *Porphyromonas*, *Rothia*, *Neisseria*, *Fusobacterium*, **Lactobacillus**
Small intestine				
Duodenum	5–7	10^{3-4}	*Escherichia coli*, *Klebsiella*, ***Enterococcus***, *Bacteroides*, *Ruminococcus*, *Dorea*, *Clostridium*, *Coprococcus*, ***Weissella*** , ***Lactobacillus***, ***Bifidobacterium***	*Granulicatella*, ***Streptococcus*** (e.g. ***S. mitis***), *Veillonella*, ***Lactobacillus***, ***Bifidobacterium***
Jejunum	7–9	10^{4-5}		
Ileum	7–8	10^{8}		
Large intestine				
Colon	5–7	10^{11}	Hundreds of species belonging to the five major phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Proteobacteria*	Dominated by autochthonous microbes

Data source Walter et al. (2001), Kerckhoffs et al. (2006), Macfarlane and Dillon (2007); LAB genera are in bold font

Table 6.6 Common LAB genera and their association with the human GIT

Family	Genus	Common human GIT member	Clinical significance and human GIT association	Reference
Aerococcaceae	*Aerococcus*	Rarely	An *Aerococcus* strain was detected in colonic biopsy of one healthy individual by molecular method. *A. urinae* may reside in human GIT and cause rare urinary tract pathogen and endocarditis	Nielsen et al. (2003), Christensen et al. (2006), Ebnöther et al. (2002)
Carnobacteriaceae	*Carnobacterium*	No	Two reported clinical cases in the pus and gangrene site of patients, which involved the species *C. maltaromaticum* and *C. divergens*	Xu et al. (1997), Chmelar et al. (2002), Leisner et al. (2007)
Enterococcaceae	*Enterococcus*	Yes	*Enterococcus* are normal human GIT commensals, but some species, e.g., *E. faecalis* and *E. faecium*, are known to cause urinary tract infections, bacteremia, and endocarditis. This group also poses significant clinical concern of antibiotic resistance	Donskey (2004), Hammerum (2012)
	Tetragenococcus	No	So far, only a *T. solitarius* strain was isolated from human ear secretions	Collins et al. (1989)
	Vagococcus	No	*Vagococcus fluvialis* was rarely isolated from clinical samples including wound, blood, and peritoneal fluid. Clinical significance remains unknown	Teixeira et al. (1997)
Lactobacillaceae	*Lactobacillus*	Yes	*Lactobacillus* are present throughout the human digestive tract, from oral cavity to intestine. At least 17 different species are associated with human GIT. They are relatively abundant in the human GIT associated with the mucosal lining, but relatively low in fecal samples (only 0.2–1.0 %). In the oral cavity, they are two-sided sword that protects against harmful bacteria, meanwhile produce acids and contribute to dental erosion. Lactobacilli are generally considered as probiotic bacteria	Mueller et al. (2006), Walter (2008), Gorkiewicz et al. (2013), Yang et al. (2010)

(continued)

Table 6.6 (continued)

Family	Genus	Common human GIT member	Clinical significance and human GIT association	Reference
	Pediococcus	Yes	*Pediococcus* strains have been isolated in healthy human saliva, feces, and colonic biopsy. *P. pentosaceus* and *P. acidilactici* were isolated from clinical specimens of blood, diarrheal stools, peritoneal fluids, abscesses, urine. Their role in pathogenesis is unsure. However, there were sporadic cases of *Pediococcus*--related bacteremia, as well as *P. acidilactici*-associated endocarditis (only 1 reported case), and *P. parvulus*-related bacteremia in a metastatic testicular cancer patient (only 1 reported case)	Walter et al. (2001), Huys et al. (2011),Sanchez et al. (2010), Barros et al. (2001), Figueroa Castro et al. (2010), Iwen et al. (2011)
Leuconostocaceae	*Leuconostoc*	Yes	This is rarely carried by gut/mucous membranes of human. *L. mesenteroides* (more prevalent) and *L. argentinum* was positively detected in fecal samples by molecular methods. *L. citreum* was detected in colonic biopsy of healthy human. Some members are related to human infections, though usually a predisposing factor is present, e.g., patients undergoing vancomycin therapy or premature babies	Walter et al. (2001), Nielsen et al. (2003), Huys et al. (2011), Sanchez et al. (2010), Heilig et al. (2002)
	Oenococcus	No	Not reported	
	Weissella	Yes	*W. cibaria* and *W. confusa* were also isolated from human clinical and gut mucosal biopsy specimens. *W. confusa* was however associated with bactermia and endocarditis rarely. *Weissella*-associated diseases are usually associated with underlying causes like vancomycin resistance, and host immunosuppression	Walter et al. (2001), Björkroth et al. (2002), Olano et al. (2001), Harlan et al. (2011), Flaherty et al. (2003)

(continued)

Table 6.6 (continued)

Family	Genus	Common human GIT member	Clinical significance and human GIT association	Reference
Streptococcaceae	*Lactococcus*	No	*Lactococcus garvieae*, a low virulent opportunistic pathogen, rarely caused endocarditis and other complications. Although immunocompetent hosts might also be susceptible, most patients were elderly with disease/predisposing factors, e.g., prosthetic heart valve, GIT lesions (ulcers, polyps), antacid drug treatment	Li et al. (2008), Russo et al. (2012)
	Streptococcus	Yes	A number of species are human GIT inhabitants reported to be residing at different body sites, including oral cavity (*S. mitis, S. salivarius*) and intestine (*S. equinus, S. salivarius, S. anginosus, S. faecalis*). The pyogenic and anginosus groups are considered as human pathogens and opportunistic pathogens, respectively	Tagg et al. (2011)
Bifidobacteriaceae	*Bifidobacterium*	Yes	*Bifidobacterium* are generally considered beneficial. They are among the first colonizers of human infant gut, but decline with age. However, *B. scardovii* and *B. dentium* were originated from human blood and dental caries, respectively. Their sources might reflect their potential pathogenic role. In colonoscopic biopsy tissue samples, the 6 major culturable bifidobacterial taxa were *B. longum*, *B. pseudocatenulatum*, *B. adolescentis*, *B. pseudolongum*, *B. breve*, and *B. bifidum*. In contrast, *B. dentium* and *B. animalis* subp. *lactis* were dominating in the fecal samples. Therefore, the composition of mucosal-attaching population differed from the fecal one. A high intra-individual variation was also noted	Turroni et al. (2009), Mantzourani et al. (2010), Claesson et al. (2011)

6.2.3 LAB in the Gut

Of the over 20 lactic acid bacterial genera, at least seven are commonly associated with the human GIT. *Bifidobacterium*, *Lactobacillus*, *Enterococcus* and *Streptococcus* are the predominant LAB genera present in the human GIT, whereas *Weissella*, *Pediococcus* and *Leuconostoc* are believed to be less prevalent (see Table 6.6).

6.2.3.1 *Lactobacillus* and *Bifidobacterium*

The study of gut *Lactobacillus* and *Bifidobacterium* has long been a main research focus in the field because of their potential probiotic effects to the host, and their prevalence in the human GIT. Moreover, it is believed that there are conspicuous developmental changes in the diversity and abundance of *Bifidobacterium* (i.e., early colonization in neonates, predomination in infants, and reduction in the elderly). A decline in the abundance in *Bifidobacterium* in the elderly are possibly linked to undesirable health consequences. Moreover, in some clinical situations, the abundances of these two genera are altered. For instance, in infant and children coelic disease, the *Lactobacillus* and *Bifidobacterium* amounts were reduced (Di Cagno et al. 2011; De Weerth et al. 2013). The relative abundance of fecal *Bifidobacterium*, but not *Lactobacillus*, was significantly lower in gout patients compared to healthy individuals (unpublished data from The Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P. R. China).

The colonization of *Lactobacillus* and *Bifidobacterium* in human gut started as early as the neonate state. Generally, it is thought that human babies are born sterile and the microbial colonization starts during the passage through the birth canal of mothers. Some of the primary colonizers include facultative anaerobes like proteobacteria, which are important in adjusting the initially oxidative environment inside the infant gut to allow the later colonization of the true anaerobic successors (e.g., *Bacteroides* and *Bifidobacterium*) (Sommer and Bäckhed 2013). The gut microbiota structure is deemed to be less complex in infants than adults (Favier et al. 2002). This view was confirmed by the recent metagenomic study by Turroni et al., which found that the three most abundant bacterial classes in infant fecal samples were *Bifidobacteriales* (80.6 %), *Lactobacillales* (7.2 %), and *Clostridiales* (3.1 %), respectively. The infant gut microbiota was largely conserved with a high proportion of *Bifidobacteriaceae* (74–99.3 %). Although the genus *Bifidobacterium* was commonly shared between the sampled infants with dominant species, *B. longum* (56.2 %) and *B. bifidum* (10.7 %), a high variation was observed in their relative abundances. For instance, *B. longum* ranged between 21.7 and 90.6 %, whereas *B. breve* ranged from 0.3 to 44.4 % of sequence reads (Turroni et al. 2012).

Several environmental factors play major roles in governing the gut microbiota of infants, including the mode of delivery (vaginal delivery vs. cesarean section),

diet, and prophylactic antibiotic (Westerbeek et al. 2006). The delivery mode had a strong influence on the early diversity and development of the gut LAB. An elegant study demonstrated that vaginally delivered infants harbored microbiota was similar to that of their own mother's vagina (mainly colonized by *Lactobacillus*, *Prevotella*, or *Sneathia*), while common skin microflora, like *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*, prevailed in cesarean section infants (Dominguez-Bello et al. 2010). Moreover, both the gut microbiota diversity and *Bifidobacterium* abundance of cesarean section neonates diminished compared to the vaginally delivered ones (Biasucci et al. 2008, 2010).

The diversity of LAB increased after birth, with *Lactobacillus* detected from the third week accompanied with frequent species change. The bifidobacterial population was rather stable until the start of weaning (José Pozuelo De Felipe et al. 2005). A similar natural *Lactobacillus* succession was observed in other studies (Heilig et al. 2002; Ahrné et al. 2005). During 1 week to 6 months, up to 45 % of infants were mainly colonized with *L. rhamnosus* and *L. gasseri*. However, other food-related species (*L. paracasei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii*) gradually succeeded after solid food introduction (Ahrné et al. 2005). The choice of breast- or formula-fed of the infants substantially affected the gut microbiota. Breast-fed infants were more heterogeneous in fecal microbial diversity (Schwartz et al. 2012). Human milk contains complex oligosaccharides, which act as substrates for the gut microbiota of breast-fed infants and selectively enrich the responsive microbial populations, in particular, the *Bifidobacterium*. Genomic analyses of the dominant species in infant gut, *B. longum* subsp. *infantis* and *B. bifidum*, revealed the presence of carbohydrate-catabolizing gene sets targeting for the human milk oligosaccharides (Sela et al. 2008; Zivkovic et al. 2011).

The infant gut microbiota gradually evolves into a more stable and adult-like structure by 1–2 years of age, and gains a high stability over time (Sommer and Bäckhed 2013). *Bifidobacterium* together with other genera of the *Bacteroidetes* and *Firmicutes* phyla form part of the stable core community of permanent gut colonizers in a long-term study monitoring over 10 years (Rajilić-Stojanović et al. 2013). The study also found that only the microbial abundance but not species diversity was affected by environmentally introduced changes (by antibiotics administration, changes in diets, distant traveling) throughout adulthood. On the other hand, owing to the change of diet, lifestyle, gut physiology, immunity and medical treatments, the gut microbiota in the elderly often differed from that of the younger adults (Biagi et al. 2012; Drago et al. 2012). Most obviously, the *Bifidobacterium* quantity and diversity declined in the gut of the elderly (Hopkins et al. 2002; Duncan and Flint 2013), especially in the cases of antibiotic treatment, hospitalization, and *Clostridium difficile*-associated diseases (Biagi et al. 2012). However, conflicting data from some recent studies suggest that the gut bifidobacterial populations in the elderly may be more abundant and diverse than previously thought (Mueller et al. 2006; Drago et al. 2012; Lahtinen et al. 2008; Biagi et al. 2010).

In contrast to the high degree of temporal stability of the autochthonous human gut residents (e.g., *Bifidobacterium* and the *Bacteroides fragilis*), the *Lactobacillus*

genus is less resilient and fluctuates a lot. The fecal *Lactobacillus* group showed considerable variation even within a 2-week period (Vanhoutte et al. 2004). Similarly, in Maukonen et al., the fecal *Lactobacillus* profiles in all the monitored subjects were fairly unstable even though a relatively high species similarity of 69.1 ± 7.3 % was shared in the studied cohort (Maukonen et al. 2008). At least 17 *Lactobacillus* species are related to the human GIT (Walter 2008). Experimental evidence, however, shows that only some of these species (e.g., *L. gasseri, L. crispatus, L. reuteri, L. salivarius, and L. ruminis*), but not the typically food-associated ones (e.g., *L. casei, L. delbrueckii, L. brevis, and L. plantarum*), are capable of persistently settling in the human GIT. Therefore, the latter species are possibly allochthonous (Walter et al. 2001; Reuter 2001) acquiring from ingested foods or other environmental source.

6.2.3.2 *Streptococcus*

Streptococcus is a broad genus consisting of about 67 species (Felis et al. 2009). Similar to *Lactobacillus* and *Bifidobacterium*, the colonization of *Streptococcus* started as early as the neonate stage. Along with *Lactobacillus* and *Bifidobacterium*, they are often detectable in relatively high abundance in infant gut samples (Magne et al. 2006). Solís et al. (2010) analyzed fecal samples of 20 vaginally delivered breast-fed infants. Both the genera of *Enterococcus* and *Streptococcus* were frequently isolated in 1-day-old newborns, and bifidobacterial species only gradually dominated from 10 days of age until 3 months old. Interestingly, in very low birth weight infants, streptococci and enterobacteria dominated for slightly longer before a retarded bifidobacterial colonization (first appearance on around 10 days after birth, and not became the prevailing genus until around 20 days post delivery) (Sakata et al. 1985). Apart from the fecal samples, the genus *Streptococcus* was reported to be present in the small intestine by a recent metagenomic study using pyrosequencing technique (Barrett et al. 2013). As expected, the ileal and colonic fluids of two infants were dominated with *Bifidobacterium* and *Lactobacillus*. However, surprisingly, along the sampling time points, these populations were not stable and could largely reduce in quantity. The reduction was accompanied with an increased proportion of *Streptococcus* and *Enterobacteriaceae*, indicating that the latter organisms might be competing for the same ecological niche with *Bifidobacterium* and *Lactobacillus*.

One possible source of *Streptococcus* for the infant gut is the mother's milk (Martín et al. 2003). Human milk contains a variety of microbes, which are likely acting as "inocula" for the infant GIT and impacting on the colonization of the gut microbial members. Streptococci along with staphylococci and LAB are frequently isolated from human breast milk (Collado et al. 2009). Ward et al. profiled the microbial 16S rRNA of ten human milk samples. Around 99 % of the bacterial population belonged to the *Proteobacteria* (65 %) and *Firmicutes* (34 %) phyla, mainly comprised of three genera, namely, *Pseudomonas* (61.1 %), *Staphylococcus* (33.4 %) and *Streptococcus* (0.5 %) (Ward et al. 2013). In another study,

the milk microbiome of most sampled mothers (n = 16) was dominated with the genera *Streptococcus* and *Staphylococcus* (Hunt et al. 2011).

In adults, the resident microbiota of the small intestine is thought to be relatively simple and of lower density as compared to the colon. Phylogenetic analysis by clone library revealed that the small intestine was prevailed by *Streptococcus*, *Escherichia coli*, *Clostridium* and high GC ratio organisms. Although commonly present, the *Streptococcus* genus was temporally unstable, as variation was shown with samples taken with a year gap (Zoetendal et al. 2012). In the human ileostomy effluents and small intestine mucosal biopsy tissues, a relatively high abundance of *Streptococcus* was detected (Wang et al. 2005; Booijink et al. 2010; Hong et al. 2011). In Booijink et al. (2010), the dominant species were further confirmed to be *S. bovis*-related.

Within the genus, the only species that is used in fermentation of yogurt is *S. thermophilus*. It has a long history of food use and is generally recognized as safe (GRAS). Some other species within this genus are of potential clinical concerns (*S. pyogenes*, *S. agalactiae*, and *S. pneumoniae*). For example, the group B streptococci (like *S. agalactiae*) are considered as normal commensals colonizing the human GIT or genital tract of healthy women. However, in clinical situation, virulent *S. agalactiae* strains may sometimes cause life-threatening infection to the newborn and the mother. Nevertheless, the probiotic properties and potential pathogenicity of this genus have been studied to some extent.

O'Shea et al. (2009) identified a novel *S. salivarius* strain from the mammalian intestine. This strain was unique in producing 3 class II bacteriocins, including 1 enterocin A and 2 salivaricin P-like bacteriocins. The application of the oral commensal, *S. salivarius* K12 strain, as probiotics has recently been proposed. *S. salivarius* possesses antibacterial activity against the opportunistic pathogen *S. pyogenes*, and may help protect from bacteria-based otitis media, halitosis, and dental caries (Wescombe et al. 2012). Another oral commensal species, *S. cristatus*, was studied for its potential immunomodulatory effect. It was able to suppress the stimulation of interleukin-8 (a potent neutrophil chemotractant that causes inflammatory response) production by the oral pathogen, *Fusobacterium nucleatum*, when both species were co-incubated with oral epithelial cells (Zhang et al. 2008).

The common yogurt species, *S. thermophilus*, was able to exert a dose-dependent and specific protective anti-inflammatory effect to inhibit lipopolysaccharide-induced TNF-α secretion (71 % inhibition by *S. thermophilus* vs. 21–32 % by other commensal bacteria) (Ménard et al. 2004). The co-incubation of live *L. acidophilus* and *S. thermophilus* on intestinal epithelial cells protected them from invasion by pathogenic *Escherichia coli*. Therefore, these probiotic bacteria together are of potential to act as protective barrier to exclude naive epithelial cells from external pathogens (Resta-Lenert and Barrett 2003). Antibiotic-associated diarrhea is due to the dysbiosis of the gut microbiota, and commonly involves an excessive growth of harmful bacteria like *Clostridium difficile*. Administration of viable *S. thermophilus* was protective against *C. difficile* infection by reducing 46 % of weight loss in mice (Kolling et al. 2012). Moreover, less amount of toxin

was detected in the cecal content with milder pathology and diarrhea in the treatment group. In contrast, more lactate was found in the cecal content. Lactate was able to suppress in vitro *C. difficile TcdA* (involves in toxin A production) gene expression. Thus, *S. thermophilus* might be able to confer beneficial effect by reducing pathogen toxin and reverting dysbiosis.

On the other hand, several emerging clinical concerns are raised regarding the probably harmful effects of gut-residing streptococci. Few lines of experimental evidence have together pointed to the possible involvement of *Streptococcus* in colorectal cancinogenesis. *Streptococcus hansenii* together with *Bacteroides vulgatus*, *Eubacterium* spp., *Ruminococcus* spp., *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* were more prevalent in the high-risk population for colorectal cancer (Moore and Moore 1995). Similarly, in another report, the abundance of *Streptococcus* along with other genera *Enterococcus*, *Escherichia*, *Shigella*, *Klebsiella*, *Streptococcus*, and *Peptostreptococcus* significantly increased in colorectal cancer patients (Wang et al. 2012). Moreover, colorectal tumor and *S. bovis* bacteremia co-occur in 25–80 % cases. One possible carcinogenic mechanism of *S. bovis* was the triggering of human IL-8 and prostaglandins E2 release by its cell wall extract, which was previously demonstrated in a caco-2 cell model. These molecules promoted preneoplastic lesions in rats, and were possibly the effectors for subsequent cancer formation (Biarc et al. 2004; Abdulamir et al. 2011).

Periodontal diseases are linked to atherosclerosis, indicating oral-residing microbiota may play a role in promoting atherosclerosis. Using a metagenomic approach, Koren et al. (2011) profiled the bacterial populations in atherosclerotic plaque, oral and gut samples of 15 patients and healthy subjects. Members of *Streptococcus* were identified in the majority of the atherosclerotic plaque, and that the abundances of *Streptococcus* in the oral cavity and the atherosclerotic plaque were positively correlated. These suggested that *Streptococcus* from the oral cavity, and possibly even the gut, may participate in the process of atherosclerosis, though the exact role needs to be further established.

6.2.3.3 *Enterococcus*

This genus was not formed until 1984, when the two species, then *Streptococcus faecalis* and *S. faecium*, were taxonomically regrouped and reassigned as *Enterococcus* (Schleifer and Kilpper-Balz 1984). Currently, there are at least 37 described species within the genus (Franz et al. 2011). *Enterococcus* are widely distributed in the environment and are associated with soil, plant, water, GIT of human and animals, rumen, and silage (Lauková 2011). Owing to their capacity in bacteriocin production, some members of this group (e.g., *E. faecium* strain K77D, *E. faecium* strain M74) have been developed as probiotics or starter cultures in food industry (Lauková 2011; Frank et al. 2007). In healthy human, the genus *Enterococcus* is considered as gut normal flora, and they can also be isolated from the vagina, oral cavity, and skin (Murray 1990).

Along with *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, *Enterococcus* are frequently reported to be one of the earliest human GIT colonizers of infant gut (Magne et al. 2006; Hopkins et al. 2005). Kirtzalidou et al. performed a large-scale screen for enterococci in 263 infant fecal samples collected on day 4, 30, and 90 after birth, and the most common isolate was *E. faecalis* followed by *E. faecium* (Kirtzalidou et al. 2012). Magne et al. also isolated enterococci (*E. faecium*, *E. faecalis* and *E. gallinarum*) from 75 % of the sampled infant feces (Magne et al. 2006).

In normal situation, *Enterococcus* exist in a relatively small quantity in human gut (Eckburg et al. 2005), and do not seem to pose any medical concern, as this genus displays low virulence and is a common resident in healthy humans. However, lately *Enterococcus* have turned into the leading nosocomial pathogens of the bloodstream, urinary tract, surgical wounds, and other sites (with a high mortality rate of 61 %) (De Fátima Silva Lopes et al. 2005; De Been et al. 2013; Gilmore et al. 2013). The two most common enterococcal human gut residents, *E. faecium* and *E. faecalis*, represent 80–90 % and 10–20 % of the clinical isolates, respectively (Murray 1990).

In addition, numerous reports found an increased abundance of *Enterococcus* in human gastrointestinal diseases, including colorectal cancer (Wang et al. 2012; Balamurugan et al. 2008; Chen et al. 2013), cirrhosis and hepatic encephalopathy (Bajaj et al. 2012; Liu et al. 2012), and Crohn's disease (Golińska 2013). Bajaj et al. (2012) pointed out that an increase in *Enterococcus* population was linked to local inflammation and reduction of the autochthonous colonic microbiota. *Enterococcus* strains isolated from Crohn's disease patients exhibited strong cell adhesion property with increased frequency of surface aggregating protein (*asa1*) and gelatinase (*gelE*) genes as compared to those from healthy subjects (Golińska 2013). Furthermore, some *Enterococcus* strains possess virulent factors like hyaluronidase (associated with tissue damage), cytolysin (beta-haemolytic), extracellular surface protein (associated with biofilm formation and endocarditis), and aggregation factor (prevent from lysosomal fusion) (Fisher and Phillips 2009). These together points to the potential pathological role and virulence of certain disease-associated *Enterococcus* strains, and that this genus is not as harmless as previously thought.

Antibiotics are widely applied to combat bacterial infection in clinics. However, the use of antibiotics may select for gut microflora like *Enterococcus*, which can readily take in new genetic traits, e.g., antibiotic resistance (De Been et al. 2013; Gilmore et al. 2013). For example, a typical complication for allogeneic hematopoietic stem cell transplant patients is bacteremia, which can be triggered by an antibiotic-associated bacterial intestinal domination (defined as the predomination of over 30 % by a single bacterial taxon). The genus *Enterococcus* is one of the most common intestinal dominators with the risk of enterococcal domination elevated by three times with metronidazole treatment, and enterococcal domination further raises the chance of vancomycin-resistant enterococcal bacteremia by nine times (Taur et al. 2012). A cirrhotic patient contracted spontaneous bacterial peritonitis with infection by the unusual species, *Enterococcus*

hirae, after receiving norfloxacin for a prophylactic treatment against *Klebsiella*. *E. hirae* causes diseases usually in animals but not human, however, in this case, it acted as a human opportunistic pathogen (Sim et al. 2012). Some other atypical human infection-related enterococci include *E. gallinarum*, *E. avium*, *E. raffinosus*, *E. hirae*, *E. mundtii*, *E. casseliflavus*, and *E. durans* (Prakash et al. 2005; Dombrádi et al. 2012). A recent study analyzed 16 fecal samples from infants with no previous exposure to antibiotics (Zhang et al. 2011). Antibiotic resistant bacteria (species in the genera *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Streptococcus*, and *Escherichia coli/Shigella*) and genes (*tet(M)*, *ermB*, *sul2*, and *blaTEM*) were detectable in infant GIT as soon as the first week after delivery. Moreover, the antibiotic gene multiplied within the host even subjected to no antibiotic selection pressure. Therefore, the human GIT may be considered as a natural gene pool for antibiotic resistance.

In view of the rising medical concerns regarding the virulence of *Enterococcus* and the risk of spreading antibiotic resistance genes, as well as the therapeutic challenge posed by the increasing clinical cases of infection by multi-drug resistance and atypical species/strains, the practice of applying *Enterococcus* in food industry and as probiotics is currently under debate (Franz et al. 2011).

6.2.3.4 *Weissella*, *Pediococcus,* and *Leuconostoc*

Pediococcus, *Leuconostoc*, and *Weissella* share similar natural habitats, which are on raw or processed foods, as well as in the human and animal guts (Huys et al. 2011). Both *Pediococcus* and *Leuconostoc* have a long history of food use because of their well-known probiotic properties. Similar to the previously described genera, these three are known to associate with the human GIT. However, there are significantly fewer reports describing their relationship with the human GIT. This is possibly due to their relatively low abundance in human fecal samples.

Weissella is a comparatively new genus split originally from *Leuconostoc* (Collins et al. 1993). They are occasionally detected in healthy human intestine tissues; therefore, it is viewed as a minority normal gut microflora in some individuals. Contrary data from a pyrosequencing study of colonic mucosal tissue revealed that *Bacteroides*, *Leuconostoc*, and *Weissella* ranged as much as 4.6–41.2 % in over 90 % of the analyzed samples (Hong et al. 2011). The presence of *Weissella* and *Leuconostoc* in human colonic tissue was further supported by Gorkiewicz et al. (2013) that monitored the change of microbiota composition in the stool and colonic biopsy samples during an osmotic-induced diarrhea by a metagenomic approach. As expected, a high intra-individual variation was observed in the fecal samples, but the spectra of microbiota in the stool and colon samples were distinct. *Weissella*, *Leuconostoc*, and *Lactobacillus* were the prevailing genera in the colonic specimens. The two most abundant and "undisturbed" phylotypes present in the colonic specimens after the induced diarrhea belonged to *W. confusa* and *W. cibaria*, and both of them were rarely detected or completely absent in the stool samples (Gorkiewicz et al. 2013). These studies

have provided clear evidence that *Weissella*, and possibly, *Leuconostoc* are autochthonous human GIT residents, at least in some individuals. Moreover, in defining the indigenous or transient members of the gut microbiota, the choice of sample is critical. Currently, most studies are based on fecal materials rather than mucosal/colonic specimens, as it is noninvasiveness to obtain fecal samples. Some *Weissella* members, mainly *W. confusa* and *W. cibaria*, have occasionally been reported in clinical and healthy human feces (Walter et al. 2001; Björkroth et al. 2002). *W. confusa* has also acted as a vancomycin-resistance opportunist and caused sporadic cases of bacteremia (Olano et al. 2001; Harlan et al. 2011; Kumar et al. 2011; Salimnia et al. 2011; Lee et al. 2011) and life-threatening endocarditis (Flaherty et al. 2003; Shin et al. 2007).

Leuconostoc are sometimes detected in human fecal samples with *L. mesenteroides* most frequently found (Walter et al. 2001; Heilig et al. 2002). Additonally, Nielson et al., identified *L. argentinum* in human feces. *Leuconostoc* are thought to be rare in human intestine (Huys et al. 2011). However, conflicting data are provided by Hong et al. and Gorkiewicz et al. that *Leuconostoc* existed in relatively high abundance (Gorkiewicz et al. 2013; Hong et al. 2011). The species, *L. citreum*, was also found in the colonic biopsy samples of around 25 % of healthy humans (Nielsen et al. 2003; Sanchez et al. 2010), and 10 % of active coeliac patients (Sanchez et al. 2010). *Leuconostoc* are usually considered safe to use in food production, but in rare occasions they are associated with human diseases including bacteremia, catheter-associated infections, sepsis, meningitis, pneumonia, urinary tract infection, osteomyelitis, and hepatic dysfunction. Short bowel syndrome, patients with gastrostomy undergoing enteral feeding, and children or neonates with underlying gastroenteral pathology may be more susceptible (Bernaldo de Quirós et al. 1991; Espinoza et al. 1997; Monsen et al. 1997; Jofré et al. 2006; Janow et al. 2008; Yossuck et al. 2009; Shin et al. 2011; Ishiyama et al. 2011; Florescu et al. 2008).

The genus *Pediococcus* is composed of the core species, *P. acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. dextrinicus*, and *P. damnosus* (Bosley et al. 1990). The two species, *P. acidilactici* and *P. pentosaceus*, have been widely used in food fermentation (e.g., dry sausage production), and are considered as human gut commensals. They have also been isolated from the saliva, fecal, and respiratory tract samples of healthy humans, as well as from clinical specimens (including blood, stools, abscesses, urine, wounds, and peritoneal fluids) (Walter et al. 2001; Barros et al. 2001; Sarma and Mohanty 1998). *P. acidilactici* is more frequently isolated in clinical samples, and is rarely associated with clinical complications like bacteremia (in elderly, infant with congenital jejunoileal atresia, pregnant woman, patient with gallbladder metastatic adenocarcinoma, who in most cases underwent antibiotic treatment) (Mastro et al. 1990; Suh 2010), hepatic abscess (Sire et al. 1992) and pneumonitis (Sarma and Mohanty 1998). However, their roles in disease pathogenesis and potential as opportunistic pathogens in healthy individuals are not entirely clear.

All three genera are considered as normal human GIT commensals and are of very low risk in causing diseases in healthy individuals. In some occasions, they

may play protective roles in the human GIT. For example, *Weissella* species were detected less frequently in duodenal biopsy samples of active than in treated coeliac children (10 % vs. 50 %) (Sanchez et al. 2010). In addition, it was observed that *P. acidilactici* along with other lactobacilli were detectable only in fecal samples of patients in remission, but not at the active inflammation phase of UC (Bullock et al. 2004). However, only very few studies have focused specifically on the role and interaction of these three genera with the human GIT, whether these LAB members offer any protective function or just exist coincidentally at the site remains to be further investigated.

Through the observation of collective clinical cases, an elevated risk of infection seems to link with particular health conditions or underlying diseases of the host (e.g., immunocompromised due to drug treatment, cancer, pre-existing GIT symptoms, preterm infants, elderly, pregnancy), and, in many cases, with the administration of antibiotics. All the three genera are associated with the human GIT, and based on the most recent metagenomic studies performed on human gut biopsy tissues, they possibly exist as autochthonous residents that presumably form close interaction with and strongly adhere to the intestinal mucosal surface. The most common clinical problem associated these genera is bacteremia. Hence, one speculated scenario is that some of these underlying predisposing factors potentially alter the GI mucosa or the intestinal integrity before certain gut microbes could gain access to the bloodstream, ultimately lead to bacteremia and further complications. In addition, the presence of vancomycin-selective pressure preferentially enriches these naturally vancomycin-resistant genera, and such dysbiosis highly increases the risk of human diseases. The fact is that these genera do reside in the pool of human GIT microbiota regardless of the host health status, and that they may cause diseases though sporadically, the clinical significance of these genera and their role as antibiotic-associated opportunistic pathogens should not be overlooked.

6.2.4 Clinical Evidence Showing Impact of LAB on Human Health

Many genera of the LAB have been considered as probiotics as they are thought to produce beneficial health effects, especially *Bifidobacterium* and *Lactobacillus* (Foligné et al. 2013; Howarth and Wang 2013; Bienenstock et al. 2013). Data from a number of human clinical trials of oral consumption of LAB have provided solid empirical evidence supporting such claim. Both *L. plantarum* P-8 (*Lp*-8) and *L. casei* Zhang (*Lc*Z) are novel probiotic bacteria isolated from traditional Chinese fermented dairy products by the Key Laboratory of Dairy Biotechnology and Engineering (Ministry of Education, P. R. China). A 4-week oral consumption of either strain by human subjects was able to elevate the fecal short-chain fatty acid (including acetate and propionate) level, reduce the concentration of the potentially carcinogenic fecal total bile acids, and modulate the gut bacterial content at

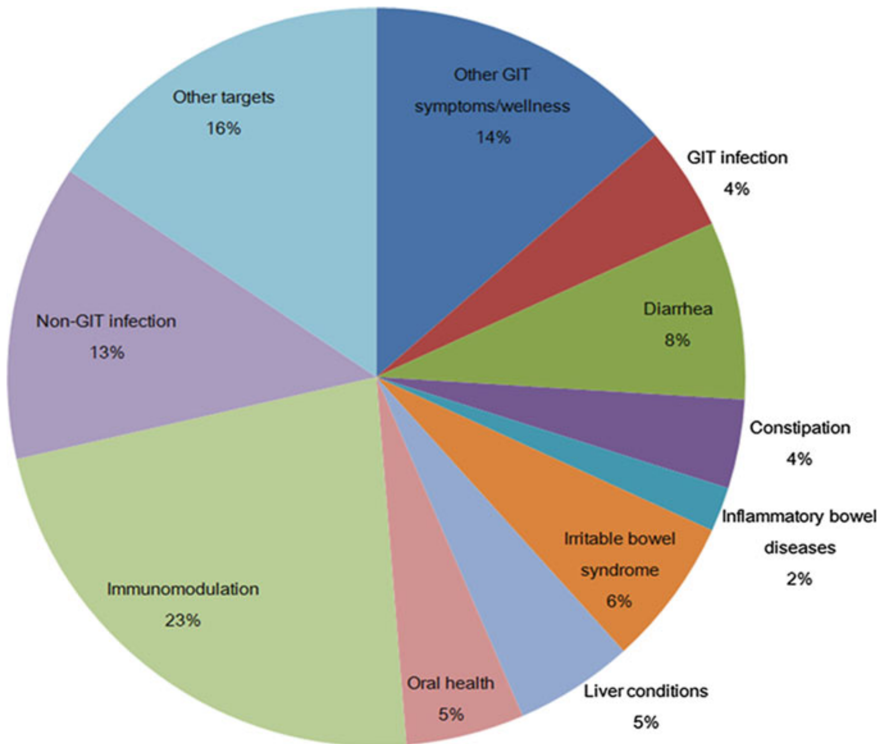


Fig. 6.3 Target functions/conditions in some completed LAB human clinical trials

least during the experimentation period ((Wang et al. 2013) and unpublished data), suggesting a direct interaction of the consumed LAB and the recipients.

ClinicalTrials.gov is the largest registry of clinical trials run by the United States National Library of Medicine (NLM) at the National Institutes of Health (holding over 130,000 trials from more than 170 countries in the world). A database search was performed using the criteria 'LAB | Exclude Unknown | Completed | received from 01/01/2001 to 10/31/2013'; 177 clinical trial registries were retrieved, of which 154 involved the administration of probiotics (either as supplement or in a probiotic product) to improve target functions or medical conditions. These can be broadly grouped into the following domains: (1) GIT infection, (2) diarrhea, (3) constipation, (4) inflammatory bowel diseases, (5) IBS, (6) Other GIT symptoms and general wellness, (7) liver conditions, (8) oral health, (9) immunomodulation, (10) non-GIT-related infection, and (11) other miscellaneous targets (Fig. 6.3). Further, PubMed database search based on their ClinicalTrials.gov Identifier (NCT) number returned 32 relevant publications. Results reported in these publications are summarized in Table 6.7, and over half of these

Table 6.7 Clinical effects of LAB in human trials

Target function, condition or disease	Tested LAB strain (as supplement /in probiotic product)	Subject (number of participants in the trial)	Project (study aims and monitored parameters)	Outcome	ClinicalTrials.gov identifier (NCT) number; reference
GIT symptoms	*L. casei* subsp. *DG*	Adults (210)	Mesalazine and/or *Lactobacillus casei* in the diverticular disease of the colon (to assess mesalazine and/or probiotics in maintaining remission in symptomatic uncomplicated diverticular disease (SUDD); recurrence of SUDD was defined as abdominal pain reappearance for >24 consecutive hours)	Effective (cyclic mesalazine or *L. casei* subsp. *DG* or in combination aided in maintaining SUDD remission)	Tursi et al. (2013)
GIT symptoms	*L. rhamnosus* GG	Children with IBS / functional abdominal pain (141)	Efficacy of *Lactobacillus* GG (LGG) in children with abdominal pain (to determine if LGG could relieve symptoms in children with recurrent abdominal pain; measured the overall pain after the intervention period and double-sugar intestinal permeability test before and after the treatment)	Effective (significantly reduced abdominal pain frequency and severity in IBS children; lower abnormal intestinal permeability; sustained effect likely due to gut barrier improvement)	Francavilla et al. (2010)
GIT health	*L. salivarius* CECT5713	6 months old infants (80)	Safety assessment of *Lactobacillus salivarius* CECT5713 in 6 months old children (to test safety and tolerance of the strain; antibiotic susceptibility profile and fecal Lactobacilli were analyzed; also by anthropometric evaluations)	Effective (no adverse effect was shown; fecal Lactobacilli increased with increase in butyric acid at 6 month)	Maldonado et al. (2010)
GIT infection	*L. reuteri* DSM 17938	Adult healthy (40)	Safety of *L. reuteri* in healthy adults (to assess the tolerability of daily intake; regulatory T cell proportion, expression of cytokines and peripheral blood mononuclear cell toll-like receptors 2 and 4; intestinal inflammation (fecal calprotectin)	DSM 17938 was safe and well-tolerated for a 2-month treatment; no significant immunological changes; but small but significant increase in fecal calprotectin)	Mangalat et al. (2012)
Diarrhea	A commercially available Kefir	Children in primary care, 1–5 years old (125)	Measuring the influence of Kefir on children's stools on antibiotics (MILK)(to assess the effect of the Kefir on antibiotic-associated diarrhea in children; outcome measured by diarrhea incidence upon antibiotic administration)	Ineffective (no effect on reducing diarrhea incidence nor other measured secondary outcome)	Merenstein et al. (2009)

(continued)

Table 6.7 (continued)

Target function, condition or disease	Tested LAB strain (as supplement /in probiotic product)	Subject (number of participants in the trial)	Project (study aims and monitored parameters)	Outcome	ClinicalTrials.gov identifier (NCT) number; reference
Diarrhea	*L. acidophilus* CL1285, *L. casei* LBC80R	Adult diarrhea patients (255)	Efficacy and safety study in the prevention of antibiotic-associated diarrhea (AAD) and *Clostridium difficile*-associated diarrhea (CDAD) in hospitalized adult patients exposed to nosocomial infection (to test probiotics in AAD and CDAD reduction; monitored GI symptoms)	Effective (AAD and CDAD symptoms were reduced; a dose-dependent effect was observed)	Gao et al. (2010)
Diarrhea	*L. reuteri* DSM 17938	Non-diarrhea children patients (106)	*L. reuteri* for the prevention of nosocomial diarrhea (to assess DSM17938 in protecting from nosocomial diarrhea; monitored incidence and duration of rotavirus infection, diarrhea, recurrent and chronic diarrhea; length of hospital stay; rehydration need)	Ineffective (no effect on the overall incidence of nosocomial diarrhea and rotavirus infection; no adverse effect was observed in probiotic consumption)	Wanke and Szajewska (2012)
Constipation	*L. paracasei* IMPC 2.1	Constipated patients (20)	Probiotic-enriched artichoke in functional constipation (to compare probiotic-enriched and ordinary artichokes in constipation; recorded treatment preference, symptom profile with Bristol stool chart, GI symptom rating scale questionnaire; short-chain fatty acid production, stool consistency)	Effective (80 % of patients preferred probiotic-containing artichoke; symptom relief was significantly higher with probiotic treatment)	Riezzo et al. (2012)
Irritable bowel syndrome	Duolac7S (7 bacterial probiotic species)	Adult patients with diarrhea dominated IBS (60)	Effect of herbal extract granules combined with probiotics on IBS with diarrhea (monitored IBS pain and discomfort relief, IBS symptoms with visual analog scale, life quality, intestinal permeability, gut microbiota composition)	Effective (application of Duolac7S and herbal extract granule together provided synergistic effect in relieving symptoms on diarrhea-typed IBS)	Ko et al. (2011)
Irritable bowel syndrome	*L. acidophilus* NCFM, *B. lactis* Bi-07	Adult patients (60)	Efficacy of probiotic bacteria in subjects with IBS or functional diarrhea/bloating (FDB) (to study effects in FBDs; checked global GI symptom relief, treatment satisfaction, change in fecal microbes and symptom severity, well-being, life quality)	Effective (bloating was improved after treatment)	Ringel-Kulka et al. (2011)

(continued)

Table 6.7 (continued)

Target function, condition or disease	Tested LAB strain (as supplement /in probiotic product)	Subject (number of participants in the trial)	Project (study aims and monitored parameters)	Outcome	ClinicalTrials.gov identifier (NCT) number; reference
Irritable bowel syndrome	*L. plantarum* MF1298	Adult patients with IBS (16)	Probiotics for IBS (to test the symptomatic effect of *L. plantarum* MF1298 on IBS patients; recorded treatment preference, number of weeks for satisfactory symptom relief)	Undesirable effect (treatment was disfavored and an unfavorable effect was reported by patients)	NCT00355810, Ligaarden et al. (2010)
High blood cholesterol	*L. paracasei* strain LPC37	Healthy and hyper cholesterolemic subjects (32)	Combination of probiotic and calcium in healthy adults (to assess the impact of probiotic supplement alone and in combination with calcium on fecal *Lactobacillus* colonization, blood low/high density lipoprotein cholesterol (LDLC, HDLC), fecal secondary bile acids (FBA))	Effective (combined treatment altered LPC37 colonization; significant FBA rise; in healthy subjects, blood LDLC, LDL/HDL ratio reduced, but moderately in patients with hypercholesterolemic)	NCT01033461, Trautvetter et al. (2012)
Metabolic syndrome and obesity	*L. casei* Shirota	Healthy (10) and metabolic syndrome (28) patients	Obesity—inflammation—metabolic disease: effect of *Lactobacillus casei* Shirota (to study *L. casei* shirota on gut permeability, endotoxin level, neutrophil function in metabolic syndrome patients; gut permeability tested by differential sugar absorption and diaminooxidase serum level; endotoxin assayed by adapted limulus amoebocyte lysate assay; LPS-binding protein, soluble CD14 assayed by ELISA; neutrophil function, toll-like receptor expression assayed by flow cytometry)	Ineffective (gut permeability of metabolic patients was significantly higher than that of the healthy controls. However, *L. casei* Shirota treatment exerted no effect on the measured parameters)	NCT01182844, Leber et al. (2012)
Oral health	*L.reuteri* DSM 17938, *L. plantarum* 299v, ATCC PTA 5289	Young healthy adults (25)	Acid production in dental plaque after exposure to probiotic bacteria (to test lactic acid production in dental plaques incubated with probiotics, and in subjects' saliva with/without taking probiotic lozenges; also measured salivary mutans streptococci/lactobacilli counts with chair-side methods)	*L. reuteri* DSM 17938 produced significantly less lactate; no evidence of increase in plaque acidity with probiotic administration	NCT01700712, Keller and Twetman (2012)
Immunity	*L. rhamnosus* GG	Healthy adults (39)	Probiotics to enhance immunogenicity of influenza vaccine in healthy adults (to test immune adjuvant effect; assayed seroconversion rate, hemagglutinin inhibition titer)	Effective, partially (*Lactobacillus* GG but not H1N1/B strains enhanced protection for strain H3N2)	NCT00620412, Davidson et al. (2011)

(continued)

Table 6.7 (continued)

Target function, condition or disease	Tested LAB strain (as supplement /in probiotic product)	Subject (number of participants in the trial)	Project (study aims and monitored parameters)	Outcome	ClinicalTrials.gov identifier (NCT) number; reference
Immunity and gut health	*B. animalis* subsp. *lactis*, BB-12, *L. acidophilus* LA-5, *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*	Healthy adults (58)	Dose response study of a fermented yogurt on the immune system and gut health (to assess the effect of consumption of this commercial yogurt drink on fecal bacterial counts in healthy adults; quantitative PCR was performed; volunteers' weight, waist girth, blood pressure, fasting plasma triglyceride and HDLC concentrations, and cholesterol/HDL-cholesterol ratio were measured)	Effective, partially (fecal *B. animalis* subsp. *lactis*, LA-5 and viable *Lactobacillus* significantly increased, whereas enterococci significantly reduced. No effect was observed on weight, waist girth, blood pressure, fasting plasma triglyceride and HDLC and cholesterol/HDL-cholesterol ratio)	NCT00730626, Savard et al. (2011)
Immunity	*L. reuteri* ATCC 55730	Infants from families with allergic disease (184)	Primary prevention of allergic disease in early child by *Lactobacillus reuteri* (to assess if perinatal/infant administration of *L. reuteri* reduced respiratory allergic diseases in school age and caused long-term side effects; measured allergic disease and skin prick test reactivity at 7 years old)	Ineffective long-term effect (no observable side effect; no reduction of respiratory allergic disease in school age; *L. reuteri* immunological protection seemed only transient)	NCT01285830, Abrahamsson et al. (2013)
Immunity	*L. reuteri*	Infants (161)	Primary prevention of allergic disease in early child by *Lactobacillus reuteri* (to assess the relationship between Th1/Th2 chemokines in infancy allergic disease, sensitization and probiotic supplementation; chemokines measured with Luminex and ELISA)	Effective, but transient (*L. reuteri* in stool in the 1st week of life, linked to low CCL17 and CCL22 (Th2); high CXCL11 (Th1) at 6 months of age; allergic diseases likely linked to high Th2-associated chemokines)	NCT01285830, Abrahamsson et al. (2011)
Immunity	*L. paracasei* ST11	Adults with allergic rhinitis (31)	Effect of a probiotic on grass pollen allergic rhinitis subjects (to assess short-term effect on allergic rhinitis nasally induced by grass pollen; assessed subjective nasal congestion; systemic and nasal immune markers e.g. IL-5, serum IgG4, IL-8, IL-10)	Effective (short-term treatment with ST11-fermented milk significantly downregulated allergic rhinitis markers e.g. nasal congestion, IL-5 and IgG4)	NCT01150253, Wassenberg et al. (2011)
Immunity	*L. rhamnosus* GR-1, a novel fecal *B. adolescentis* isolate	Adults with allergic rhinitis (36)	The anti-allergic effects of specific probiotics (to assess the effect of a new probiotic formulation on seasonal rhinitis; outcome parameters included quality of life scores, use of antihistamines, nasal lavage of eosinophil cationic protein, and serum IL-10, IL-12, TGF-beta)	Ineffective in giving clinical benefit; but showed immunomodulatory effects (no significant effect on life quality scores, antihistamine use or eosinophil cationic protein concentration; rise in sIL-10, sIL-12 in probiotic group. Significant rise in sTGF-beta in ragweed season)	NCT00480129, Koyama et al. (2010)

(continued)

Table 6.7 (continued)

Target function, condition or disease	Tested LAB strain (as supplement /in probiotic product)	Subject (number of participants in the trial)	Project (study aims and monitored parameters)	Outcome	ClinicalTrials.gov identifier (NCT) number; reference
Immunity	*L. rhamnosus GG, L. acidophilus* La-5 and *B. animalis* subsp. *lactis* Bb-12	Pregnant mother (450)	Impact in off-spring of mothers after perinatal daily intake of a probiotic (to assess if probiotics given to pregnant women could prevent atopic sensitization/allergic diseases during the child's first 2 years; children with itchy rash for >4 weeks were checked for atopic disease; all children were evaluated for atopic sensitization, atopic disease, asthma, allergic rhinoconjunctivitis; intention-to-treat (ITT) analysis done by multiple imputations)	Effective (incidence of cumulative atopic disease decreased, but the treatment had no impact on atopic sensitization)	NCT00159523, Dotterud et al. (2010)
Immunity	*L. rhamnosus* GG	Infants with atopic disease family history and wheezing episodes (131)	Prevention of asthma and allergy by probiotic *Lactobacillus* GG (to assess the impact of LGG on allergic sensitization and asthma in infants at risk; outcome measured by the need of inhalation, number of symptom-free days, IgE, eosinophils, eosinophilic cationic protein, TGF-beta)	Effective, but weak and transient (no clinical benefit on atopic dermatitis or asthma-associated symptoms; only mild effect on allergic sensitization, which lasted 6 months after treatment ceased)	NCT00490425, Rose et al. (2010)
Immunity	*L. casei* Shirota	Healthy elderly in nursing home (737)	Study of the effect of probiotics on respiratory morbidity after influenza vaccination of elderly in nursing homes (to assess respiratory symptom susceptibility reduction and immune response improvement post influenza vaccination; monitored period and incidence of respiratory symptoms, anti-influenza antibody titer by hemagglutination inhibition after vaccination)	Ineffective (no statistically significant clinical effect was shown on the protection against respiratory symptoms by a daily consumption of a *L. casei* Shirota-containing fermented milk)	NCT00849277, Van Puyenbroeck et al. (2012)
Immunity	*L. rhamnosus* GG	Infant (39)	*Lactobacillus rhamnosus* GG: interaction with human microbiota and immunity (in particular effect on skin, gut microbiota, humoral immunity in infants with atopic dermatitis; enumerated IgA and IgM-secreting cells by enzyme-linked immunospot and flow cytometry, skin and gut bacteria by PCR)	Effective (enhanced gut barrier function and aided in the development of immune responses)	NCT01148667, Nermes et al. (2011)

(continued)

Table 6.7 (continued)

Target function, condition or disease	Tested LAB strain (as supplement /in probiotic product)	Subject (number of participants in the trial)	Project (study aims and monitored parameters)	Outcome	ClinicalTrials.gov identifier (NCT) number; reference
Bactrial vaginosis	*L. crispatus* CTV-05 (LACTIN-V)	Women with bacterial vaginosis (24)	Safety and efficacy study of *Lactobacillus* administered vaginally in women with bacterial vaginosis (to evaluate the relationship between 7 bacterial vaginosis and 2 *Lactobacillus* species with LACTIN-V; quantitative PCR to enumerate 9 vaginosis-related bacterial species and *L. cripatus* in vaginal swabs)	Effective (vaginal colonization by CTV-05 observed in some subjects; those not colonized by CTV-05 had generally higher abundance of vaginosis-associated bacteria. Coloziation of CTV-05 was inversely correlated with intrinsic *L. crispatus*)	NCT00635622, Ngugi et al. (2011)
Bactrial vaginosis	*L. crispatus* CTV-05 (LACTIN-V)	Women with bacterial vaginosis (24)	Safety and efficacy study of *Lactobacillus* administered vaginally in women with bacterial vaginosis (to evaluate colonization efficiency, tolerability, safety, and acceptability of *L. crispatus* CTV-05 in women with bacterial vaginosis)	Effective (LACTIN-V colonized well; safe and acceptable in women treated for bacterial vaginosis; adverse effects were of equal rate in treatment and placebo groups)	NCT00635622, Hemmerling et al. (2010)
Bactrial vaginosis	*L. crispatus* (Lactin-V; Osel)	Premenopausal women with recurrent urinary tract infection (100)	Intravaginal LACTIN-V for prevention of recurrent urinary tract infection (to assess the protective effect of the vaginal suppository on recurrent urinary tract infection; outcome was measured by microbiological culture and quantitative PCR for urine samples and vaginal swabs for *L. crispatus*)	Effective (high level of vaginal *L. crispatus* colonization and a reduction of recurrent urinary tract infection)	NCT00305227, Stapleton et al. (2011)
Respiratory tract infection	*L. acidophilus* NCFM and *B. animalis* subsp *lactis* Bi-07	Children, 3–5 years old (326)	Evaluation of probiotics on symptoms of upper respiratory tract infections (to assess the efficacy of probiotic consumption on cold and influenza-like symptom incidence and duration in winter; outcome measured by the incidence and duration of fever, coughing, rhinorrhea absence days from school, necessity of antibiotics)	Effective (single and combination probiotics reduced fever, coughing and rhinorrhea incidence and duration, as well as days absent from group child care. Antibiotic use incidence was reduced)	NCT00599430, Leyer et al. (2009)

(continued)

Table 6.7 (continued)

Target function, condition or disease	Tested LAB strain (as supplement /in probiotic product)	Subject (number of participants in the trial)	Project (study aims and monitored parameters)	Outcome	ClinicalTrials.gov identifier (NCT) number; reference
Respiratory tract infection	*L. rhamnosus* GG	Mechanically ventilated patients at high risk of pneumonia (146)	*Lactobacillus* GG in the prophylaxis of ventilator-associated pneumonia (to assess if nosocomial infection and ventilator-associated pneumonia could be reduced by probiotic modification of the upper aerodigestive flora; monitored the incidence and duration of pneumonia, *Clostridium difficil* -associated diarrhea (CDAD), need for antibiotic reduction)	Effective (treatment reduced the incidence of contracting pneumonia and CDAD; no difference in duration of diarrhea episode; reduction of number of days of antibiotic usage/dose; no adverse effect was observed)	NCT00613795, Morrow et al. (2010)
Kidney stone formation	Oxadrop and AKSB[a]	Patients with mild hyperoxaluria (40)	Use of oral probiotics to reduce urinary oxalate excretion (to assess the effect of controlled diet and probiotics on urinary oxalate excretion; assayed urinary oxalate excretion, calcium oxalate supersaturation, fecal *lactobacilli*, enterococci, yeasts)	Ineffective (the tested probiotics did not influence urinary oxalate levels in patients)	NCT00587041, Lieske et al. (2010)
Infantile colic	*L. reuteri* DSM 17938	Infant (80)	*Lactobacillus reuteri* for the Treatment of Infantile Colic (recorded children (%) reduced daily average crying time of $\geq$ 50 %, and duration of crying (min/day) at 7, 14, 21, 28 days)	Effective (crying reduced)	NCT01046617, Szajewska et al. (2013)
Mastitis	*L. fermentum* CECT5716, *L. salivarius* CECT5713	Women with infectious mastitis (352)	Use of probiotic *Lactobacillus* for the treatment of lactational mastitis (to evaluate 2 breast milk-isolated strains in treating lactational mastitis vs. antibiotics; milk sample bacterial and *Lactobacillus* counts, and mastitis recurrent rate were measured)	Effective (either of the tested strain was efficient in treating infectious mastitis during lactation with lower recurrent rate)	NCT00716183, Arroyo et al. (2010)

[a] Oxadrop (VSL Pharmaceuticals) contains *L. acidophilus*, *L. brevis*, *S. thermophilus*, and *B. infantis*, whereas AKSB (Agri-King) contains fructo-oligosaccharide, *Enterococcus faecium* (SF68), *Saccharomyces cerevisiae* subsp boulardii, and *Saccharomyces cerevisiae*

Table 6.8 Clinical effects of the less studied LAB genera in human trials

Tested LAB strain (probiotic product or supplement)	Subject (number of participants in the trial)	Project aims	Outcome	Reference
B. animalis subsp *lactis*, *S. thermophilus*, *L. bulgaricus*, *Lc. lactis* subsp *lactis* in fermented milk	Healthy women with no GI or psychiatric symptoms (36)	To assess whether the consumption of a fermented milk with probiotics would change the brain intrinsic connectivity or responses to emotional attention tasks	Effective (the brain regions of central processing of emotion/ sensation were altered; caused by decrease in task-related response of a distributed functional network, namely somatosensory, affective, viscerosensory cortices; intrinsic activity of resting brain altered due to the change in midbrain connectivity)	Tillisch et al. (2013)
High levels of *Lc. lactis* and *Leu. mesenteroides* (Camembert cheese)	Healthy adults (12)	To evaluate if Camembert cheese microbes were detectable in fecal samples after regular consumption; effect on global metabolic activities of host gut microbiota	Ineffective (no significant effect was observed in metabolic activities, but *Leu. mesenteroides* was persistently detected until 15 days after stopping Camembert consumption)	Firmesse et al. (2008)
B. bifidum, *B. lactis*, *Lc. lactis* (Ecologic Panda)	Mother of high risk (with allergic disease family history) children during pregnancy and their children (102)	To evaluate the primary prevention of allergic disease in high-risk children by pre- and postnatal supplementation of selected probiotic bacteria	Effective (a preventive effect of eczema was observed, which persisted for the first 2 years of life)	Niers et al. (2009)
Genetically modified *Lc. lactis* (LL-Thy12) (thymidylate synthase gene replaced by human interleukin 10)	Crohn's disease patients (10)	To evaluate the effect of a transgenic human IL-10-expressing *Lactococcus* strain on Crohn's disease patients	Effective (a decrease in disease activity was resulted; transgenic bacteria were detected in feces of patients)	Braat et al. (2006)

(continued)

Table 6.8 (continued)

Tested LAB strain (probiotic product or supplement)	Subject (number of participants in the trial)	Project aims	Outcome	Reference
L. rhamnosus GG, *Lc. lactis*	Healthy adults (30)	To evaluate the immunomodulatory effect of oral intake of probiotics	Effective (significant rise in neutrophil CR3 receptor expression after coadministering *Lc. lactis* and *S. typhi* vaccine vs. placebo/ LGG alone)	Fang et al. (2000)
E. faecalis SL-5 in a topical lotion	Patients with acne	To evaluate the treatment effect on mild to moderate vulgaris acne (topical application)	Effective (significantly reduction of inflammatory lesions in patients)	Kang et al. (2009)
E. faecalis T-110, *Clostridium butyricum* TO-A, and *Bacillus mesentericus* TO-A	Patients with pancreaticobiliary diseases and underwent	pancreaticoduodenectomy (70)	To evaluate the effect of probiotics on surgical outcome after pancreaticoduodenectomy	Effective (significantly lower incidence of postoperative infectious complications in the treatment group)
Nomura et al. (2007)				
Escherichia coli (DSM 17252), *E. faecalis* (DSM 16440) in ProSymbioflor	Patients with irritable bowel syndrome (297)	To evaluate the effect of the probiotic mix in treating irritable bowel syndrome	Effective (global symptom and abdominal pain were greatly reduced in treatment group)	Enck et al. (2008)
E. faecium IS-27526 in low-fat milk	Preschool children	To evaluate the effect of *E. faecium* IS-27526 in milk on humoral immune response (total serum and total salivary sIgA) and on bodyweight	Effective, partially (significant positive effects on salivary sIgA but not total serum sIgA; significant weight gain in the treated preschool children)	Surono et al. (2011)

(continued)

Table 6.8 (continued)

Tested LAB strain (probiotic product or supplement)	Subject (number of participants in the trial)	Project aims	Outcome	Reference
E. faecium, *Bacillus subtilis*	*Helicobacter pylori* infected patients (352)	To evaluate the eradication of *Helicobacter pylori* by the probiotic treatment	Effective (treatment group receiving proton-based-inhibitor triple therapy plus probiotics showed a higher eradication rate than the proton-based-inhibitor triple therapy only group; diarrhea and overall side effects were more common in control group receiving no probiotics)	Park et al. (2007)
Living and non-living *Escherichia coli* (Symbioflor®2, SymbioPharm GmbH)	Children with irritable bowel syndrome (203)	To evaluate the effect of Symbioflor®2 in relieving symptoms of children suffering from irritable bowel syndrome	Effective (treated children showed significant symptom relief in abdominal pain, stool frequency, bloating, mucous and blood in stool, need for straining at stools, urge to defecate; the treatment was well-tolerated and of high efficacy)	Martens et al. (2010)
L. bulgaricus, *S. thermophilus*	Patients with nonalcoholic fatty liver disease (28)	To assess the effect of the tested probiotics on liver aminotransferases in nonalcoholic fatty liver disease patients	Effective (liver aminotransferases decreased in the treated patients, though cardiovascular risk factors and anthropometric parameters stayed unchanged)	Aller et al. (2011)
S. salivarius strain M18	Dental caries-active children (100)	To evaluate the effect of the probiotic *S. salivarius* strain M18 on dental health in children	Effective (significantly lower plaque scores and *S. mutans* counts for M18-treated group, particularly in subjects with high initial plaque scores)	Burton et al. (2013)

(continued)

Table 6.8 (continued)

Tested LAB strain (probiotic product or supplement)	Subject (number of participants in the trial)	Project aims	Outcome	Reference
S. salivarius (salivaricin A-producing) in milk	Children (219)	To assess the effect of consuming milk with salivaricin A-producing *S. salivarius* on SalA-like inhibitory activity on the tongue	Effective in a low proportion of subjects (20 of the 189 children showed markedly increased in SalA-like inhibitory activity after treatment)	Dierksen et al. (2007)
P. pentosaceus 5-33:3, *Leu. mesenteroides*32-77:1, *L. paracasei* subsp. *paracasei* 19, *L. plantarum* 2,362 (Synbiotic 2000Forte)	Critically ill, mechanically ventilated, multiple trauma patients (65)	To evaluate the effect on infection rate, systemic inflammatory response syndrome (SIRS), severe sepsis, mortality, stay in intensive care unit, mechanical ventilation of critically ill patients	Effective (significant reduction of infections, SIRS, severe sepsis, mortality, duration of stay in intensive care unit and mechanical ventilation for the treatment group)	Kotzampassi et al. (2006)
Tetragenococcus halophilus Th221 (an isolate from soy sauce moromi)	Patients with perennial allergic rhinitis (45)	To assess the T helper type 1 immunity promoting and perennial allergic rhinitis protective effects of *T. halophilus* Th221	Effective only in high dose group (high dose consumption significantly improved nasal symptoms, sneezing, rhinorrhea and IgE)	Nishimura et al. (2009)

B. Bifidobacterium, *E. Enterococcus*, *L. Lactobacillus*, *Lc. Lactococcus*, *Leu. Leuconostoc*, *P. Pediococcus*, *S. Streptococcus*, *S. typhi Salmonella typhi*

human trials showed promising clinical effects. Interestingly, a potential probiotic strain, *L. plantarum* MF1298, which had the optimal in vitro desirable properties among 22 other strains, led to negative symptom relief effects in IBS patients. This was the first report of an undesirable effect exerted to subjects after probiotics consumption (Ligaarden et al. 2010). These reports together clearly shows a direct health impact of LAB administration. Apart from *Lactobacillus* and *Bifidobacterium*, the probiotic potentials of other LAB genera (*Enterococcus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Tetragenococcus*) have also been studied in human but to a lesser extent (results of some recent studies are given in Table 6.8). Only minute amount of human clinical trial data are available for the genera *Pediococcus*, *Leuconostoc* and *Tetragenococcus*.

The gut microflora and LAB can exist as either autochthonous or allochthonous members, forming a relatively permanent or only a transient relationship with the human GIT. The fact that some of the allochthonous species (e.g., *L. acidophilus*, *L. casei*, *L. paracasei*, *L. rhamnosus*) (Walter 2008) are able to exert clinical effects to the host suggests that even a transient passage of these microbes to the human GIT could potentially bring about a physiological impact. Moreover, the oral consumption of probiotics is not only able to improve symptoms and disorders in the human GIT, but also alter non-GIT-related functions like enhancing hepatic health, regulating host immunity, reducing respiratory tract infection, infantile colic and mastitis (Table 6.7). This indicates that the human GIT and its functions are well connected to other organ systems. However, as reported in many studies, the clinical responses related to probiotics administration are often transient, which might cease soon after stopping the probiotics usage (Rose et al. 2010; Abrahamsson et al. 2011, 2013).

6.3 Summary and Perspectives

The human GIT is composed of a complexity of ecological environments hosting a wide array of gut microbiota adapted to the various niches. The breakthrough of the NGS technology has transformed our understanding of the structure and function of the human gut microbiota. This research field will definitely remain as a major focus in life and medical sciences in the twenty-first century. Yet, certain gaps in the knowledge need to be filled, especially the correlation between the metagenome and health/disease status, the determination of the active portion of the human gut microbiome responsible for the individual phenotypes, the functionalities of the individual microbial members and their cellular/molecular interactions with the host, as well as the characterization/role of the understudied (eukaryotic, viral, and archaeal) and the rare 'most wanted' populations within the gut microbiome.

LAB are important members of the human GIT. The ecological succession and functions of several LAB genera, namely *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Enterococcus*, have been studied extensively during the last decades

partially because of their relative dominance. Some of them are identified as autochthonous residents living in the gut throughout the human life span, although it is not always possible to clearly distinguish between the autochthonous and allochthonous members. Thanks to the advent of the metagenomic approach and an increased availability of experimental data from human intestinal biopsy tissues (vs. fecal samples), more information about the relatively less studied genera (*Weissella*, *Leuconostoc* and *Pediococcus*) and their association with the human GIT are revealed. Overall speaking, most LAB seldom cause human diseases except in high-risk individuals like preterm infants, elderly and immunocompromised patients. In many cases, LAB-associated diseases are also somewhat related to antibiotic administration, and that the human GIT as an antibiotic resistance gene pool is an emerging medical concern. On the other hand, vast amount of human clinical trial evidence supports the beneficial roles of administering these microbes to maintain and improve human health. Therefore, in consideration of the potential harmful and probiotic relationship of LAB with the human GIT and health, a balance should be striken in the design of policies concerning their applications.

The future development will lie on utilizing our knowledge, possibly with a "multi-omics" approach, to improve the quality of life, in particular by early detection and intervention of diseases. The flourishing of some of the very exciting research fields is anticipated in the near future, which include the defining of fecal microbial-based health/diagnostic markers, development of fecal transplantation therapy, exploration of novel functions and clinical usage of probiotic LAB, and an integrated application of these areas at a personalized medicine level.

References

Abdulamir AS, Hafidh RR, Abu Bakar F. The association of *Streptococcus bovis/gallolyticus* with colorectal tumors: the nature and the underlying mechanisms of its etiological role. J Exp Clin Cancer Res CR. 2011;30:11.

Abrahamsson TR, Sandberg Abelius M, Forsberg A, Björkstén B, Jenmalm MC. A Th1/Th2-associated chemokine imbalance during infancy in children developing eczema, wheeze and sensitization. Clin Exp Allergy J Br Soc Allergy Clin Immunol. 2011;41:1729–1739.

Abrahamsson TR, Jakobsson T, Björkstén B, Oldaeus G, Jenmalm MC. No effect of probiotics on respiratory allergies: a seven-year follow-up of a randomized controlled trial in infancy. Pediatr Allergy Immunol Off Publ Eur Soc Pediatr Allergy Immunol. 2013;24:556–61.

Ahrné S, Lönnermark E, Wold AE, Aberg N, Hesselmar B, Saalman R, Strannegård I-L, Molin G, Adlerberth I. *Lactobacilli* in the intestinal microbiota of Swedish infants. Microbes Infect Inst Pasteur. 2005;7:1256–62.

Aller R, De Luis DA, Izaola O, Conde R, Gonzalez Sagrado M, Primo D, De La Fuente B, Gonzalez J. Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. Eur Rev Med Pharmacol Sci. 2011;15:1090–1095.

Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nyrén P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS ONE. 2008;3:e2836.

Arroyo R, Martín V, Maldonado A, Jiménez E, Fernández L, Rodríguez JM. Treatment of infectious mastitis during lactation: antibiotics versus oral administration of *Lactobacilli* isolated from breast milk. Clin Infect Dis Off Publ Infect Dis Soc Am. 2010;50:1551–8.

Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. Nature. 2011;473:174–80.

Aziz Q, Doré J, Emmanuel A, Guarner F, Quigley EMM. Gut microbiota and gastrointestinal health: current concepts and future directions. Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc. 2013;25:4–15.

Bajaj JS, Hylemon PB, Ridlon JM, Heuman DM, Daita K, White MB, Monteith P, Noble NA, Sikaroodi M, Gillevet PM. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. AJP Gastrointest Liver Physiol. 2012;303:G675–85.

Balamurugan R, Rajendiran E, George S, Samuel GV, Ramakrishna BS. Real-time polymerase chain reaction quantification of specific butyrate-producing bacteria, *Desulfovibrio* and *Enterococcus faecalis* in the feces of patients with colorectal cancer. J Gastroenterol Hepatol. 2008;23:1298–303.

Barrett E, Guinane CM, Ryan CA, Dempsey EM, Murphy BP, O'Toole PW, Fitzgerald GF, Cotter PD, Ross RP, Stanton C. Microbiota diversity and stability of the preterm neonatal ileum and colon of two infants. MicrobiologyOpen. 2013;2:215–25.

Barros RR, Carvalho MDGS, Peralta JM, Facklam RR, Teixeira LM. Phenotypic and genotypic characterization of *Pediococcus* strains isolated from human clinical sources. J Clin Microbiol. 2001;39:1241–6.

Bernaldo de Quirós JC, Muñoz P, Cercenado E, Hernandez Sampelayo T, Moreno S, Bouza E. *Leuconostoc* species as a cause of bacteremia: two case reports and a literature review. Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol. 1991;10:505–509.

Biagi E, Nylund L, Candela M, et al. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. PLoS ONE. 2010;5:e10667.

Biagi E, Candela M, Fairweather-Tait S, Franceschi C, Brigidi P. Aging of the human metaorganism: the microbial counterpart. Age Dordr Neth. 2012;34:247–67.

Biarc J, Nguyen IS, Pini A, et al. Carcinogenic properties of proteins with pro-inflammatory activity from *Streptococcus infantarius* (formerly *S. bovis*). Carcinogenesis. 2004;25:1477–84.

Biasucci G, Benenati B, Morelli L, Bessi E, Boehm G. Cesarean delivery may affect the early biodiversity of intestinal bacteria. J Nutr. 2008;138:1796S–800S.

Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev 2010;86 Suppl 1:13–15.

Bienenstock J, Gibson G, Klaenhammer TR, Walker WA, Neish AS. New insights into probiotic mechanisms: a harvest from functional and metagenomic studies. Gut Microbes. 2013;4:94–100.

Björkroth KJ, Schillinger U, Geisen R, Weiss N, Hoste B, Holzapfel WH, Korkeala HJ, Vandamme P. Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., detected in food and clinical samples. Int J Syst Evol Microbiol. 2002;52:141–8.

Booijink CCGM, El-Aidy S, Rajilić-Stojanović M, Heilig HGHJ, Troost FJ, Smidt H, Kleerebezem M, De Vos WM, Zoetendal EG. High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol. 2010;12:3213–27.

Borrel G, Harris HMB, Tottey W, Mihajlovski A, Parisot N, Peyretaillade E, Peyret P, Gribaldo S, O'Toole PW, Brugère J-F. Genome sequence of *Candidatus* Methanomethylophilus alvus Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. J Bacteriol. 2012;194:6944–5.

Bosley GS, Wallace PL, Moss CW, Steigerwalt AG, Brenner DJ, Swenson JM, Hebert GA, Facklam RR. Phenotypic characterization, cellular fatty acid composition, and DNA relatedness of aerococci and comparison to related genera. J Clin Microbiol. 1990;28:416–21.

Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJH, Neirynck S, Peppelenbosch MP, Steidler L. A phase I trial with transgenic bacteria expressing

interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol Off Clin Pr J Am Gastroenterol Assoc. 2006;4:754–9.

Breitbart M, Haynes M, Kelley S, et al. Viral diversity and dynamics in an infant gut. Res Microbiol. 2008;159:367–73.

Brusa T, Canzi E, Allievi L, Puppo E, Ferrari A. Methanogens in the human intestinal tract and oral cavity. Curr Microbiol. 1993;27:261–5.

Bullock NR, Booth JCL, Gibson GR. Comparative composition of bacteria in the human intestinal microflora during remission and active ulcerative colitis. Curr Issues Intest Microbiol. 2004;5:59–64.

Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson WM, Hale JDF, Wescombe PA. Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. J Med Microbiol. 2013;62:875–84.

Candela M, Maccaferri S, Turroni S, Carnevali P, Brigidi P. Functional intestinal microbiome, new frontiers in prebiotic design. Int J Food Microbiol. 2010;140:93–101.

Cardona S, Eck A, Cassellas M, Gallart M, Alastrue C, Dore J, Azpiroz F, Roca J, Guarner F, Manichanh C. Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiol. 2012;12:158.

Carr FJ, Chill D, Maida N. The lactic acid bacteria: a literature survey. Crit Rev Microbiol. 2002;28:281–370.

Cecchini DA, Laville E, Laguerre S, Robe P, Leclerc M, Doré J, Henrissat B, Remaud-Siméon M, Monsan P, Potocki-Véronèse G. Functional metagenomics reveals novel pathways of prebiotic breakdown by human gut bacteria. PLoS ONE. 2013;8:e72766.

Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB. Decreased diversity of the fecal Microbiome in recurrent *Clostridium difficile*-associated diarrhea. J Infect Dis. 2008;197:435–8.

Chen HM, Yu YN, Wang JL, et al. Decreased dietary fiber intake and structural alteration of gut microbiota in patients with advanced colorectal adenoma. Am J Clin Nutr. 2013;97:1044–52.

Chmelar D, Matusek A, Korger J, Durnová E, Steffen M, Chmelarová E. Isolation of Carnobacterium piscicola from human pus—case report. Folia Microbiol (Praha). 2002;47:455–7.

Christensen JJ, Skov R. *Aerococcus urinae*. Antimicrobial therapy vaccines microbes. 2nd ed. Apple Trees Productions: New York City; 2006. p. 41–44.

Claesson MJ, Wang Q, O'Sullivan O, Greene-Diniz R, Cole JR, Ross RP, O'Toole PW. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res. 2010;38:e200.

Claesson MJ, Cusack S, O'Sullivan O, et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4586–45.

Collado MC, Delgado S, Maldonado A, Rodríguez JM. Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. Lett Appl Microbiol. 2009;48:523–8.

Collins MD, Facklam RR, Farrow JA, Williamson R. *Enterococcus raffinosus* sp. nov., *Enterococcus solitarius* sp. nov. and *Enterococcus pseudoavium* sp. nov. FEMS Microbiol Lett. 1989;48:283–8.

Collins MD, Samelis J, Metaxopoulos J, Wallbanks S. Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. J Appl Bacteriol. 1993;75:595–603.

Colson P, Fancello L, Gimenez G, Armougom F, Desnues C, Fournous G, Yoosuf N, Million M, La Scola B, Raoult D. Evidence of the megavirome in humans. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2013;57:191–200.

Cotillard A, Kennedy SP, Kong LC, et al. Dietary intervention impact on gut microbial gene richness. Nature. 2013;500:585–8.

Davidson LE, Fiorino A-M, Snydman DR, Hibberd PL. *Lactobacillus* GG as an immune adjuvant for live-attenuated influenza vaccine in healthy adults: a randomized double-blind placebo-controlled trial. Eur J Clin Nutr. 2011;65:501–7.

De Been M, van Schaik W, Cheng L, Corander J, Willems RJ. Recent recombination events in the core genome are associated with adaptive evolution in *Enterococcus faecium*. Genome Biol Evol. 2013;5:1524–35.

De Fátima Silva Lopes M, Ribeiro T, Abrantes M, Figueiredo Marques JJ, Tenreiro R, Crespo MTB. Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. Int J Food Microbiol. 2005;103:191–198.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A. 2010;107:14691–6.

De Weerth C, Fuentes S, Puylaert P, de Vos WM. Intestinal microbiota of infants with colic: development and specific signatures. Pediatrics. 2013;131:e550–8.

Demirel G, Celik IH, Erdeve O, Saygan S, Dilmen U, Canpolat FE. Prophylactic *Saccharomyces boulardii* versus nystatin for the prevention of fungal colonization and invasive fungal infection in premature infants. Eur J Pediatr. 2013a;172:1321–6.

Demirel G, Erdeve O, Celik IH, Dilmen U. *Saccharomyces boulardii* for prevention of necrotizing enterocolitis in preterm infants: a randomized, controlled study. Acta Paediatr Oslo Nor 1992a. 2013b. doi: 10.1111/apa.12416.

Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4554–4561.

Di Cagno R, De Angelis M, De Pasquale I, et al. Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization. BMC Microbiol. 2011;11:219.

Dierksen KP, Moore CJ, Inglis M, Wescombe PA, Tagg JR. The effect of ingestion of milk supplemented with salivaricin A-producing *Streptococcus salivarius* on the bacteriocin-like inhibitory activity of streptococcal populations on the tongue. FEMS Microbiol Ecol. 2007;59:584–91.

Dombrádi Z, Dobay O, Nagy K, Kozák A, Dombrádi V, Szabó J. Prevalence of *vanC* vancomycin-resistant enterococci in the teaching hospitals of the University of Debrecen, Hungary. Microb Drug Resist. 2012;18:47–51.

Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010;107:11971–5.

Donskey CJ. The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. Clin Infect Dis Off Publ Infect Dis Soc Am. 2004;39:219–26.

Dotterud CK, Storrø O, Johnsen R, Oien T. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. Br J Dermatol. 2010;163:616–23.

Drago L, Toscano M, Rodighiero V, De Vecchi E, Mogna G. Cultivable and pyrosequenced fecal microflora in centenarians and young subjects. J Clin Gastroenterol 2012;46 Suppl:S81–84.

Dridi B, Henry M, El Khéchine A, Raoult D, Drancourt M. High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. PLoS ONE. 2009;4:e7063.

Dridi B, Raoult D, Drancourt M. Archaea as emerging organisms in complex human microbiomes. Anaerobe. 2011;17:56–63.

Dridi B, Henry M, Richet H, Raoult D, Drancourt M. Age-related prevalence of *Methanomassiliicoccus luminyensis* in the human gut microbiome. APMIS Acta Pathol Microbiol Immunol Scand. 2012a;120:773–7.

Dridi B, Fardeau M-L, Ollivier B, Raoult D, Drancourt M. *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. Int J Syst Evol Microbiol. 2012b;62:1902–7.

Duncan SH, Flint HJ. Probiotics and prebiotics and health in ageing populations. Maturitas. 2003. doi:10.1016/j.maturitas.2013.02.004.

Ebnöther C, Altwegg M, Gottschalk J, Seebach JD, Kronenberg A. *Aerococcus urinae* Endocarditis: case report and review of the literature. Infection. 2002;30:310–3.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science. 2005;308:1635–8.

Enck P, Zimmermann K, Menke G, Müller-Lissner S, Martens U, Klosterhalfen S. A mixture of *Escherichia coli* (DSM 17252) and *Enterococcus faecalis* (DSM 16440) for treatment of the irritable bowel syndrome—a randomized controlled trial with primary care physicians. Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc. 2008;20:1103–9.

Espinoza R, Kusne S, Pasculle AW, Wada S, Fung J, Rakela J. *Leuconostoc* bacteremia after liver transplantation: another cause of vancomycin resistant Gram-positive infection. Clin Transplant. 1997;11:322–4.

Fang H, Elina T, Heikki A, Seppo S. Modulation of humoral immune response through probiotic intake. FEMS Immunol Med Microbiol. 2000;29:47–52.

Favier CF, Vaughan EE, De Vos WM, Akkermans ADL. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol. 2002;68:219–26.

Felis GE, Dellaglio F, Torriani S. Taxonomy of probiotic microorganisms. In: Charalampopoulos D, Rastall RA, editors. Prebiotics and probiotics science and technology. New York: Springer Sience + Business media; 2009. p. 591–637.

Figueroa Castro CE, Knezetic JA, Cavalieri SJ. Use of 16S rRNA gene sequencing to identify a case of *Pediococcus parvulus* bacteremia in a patient with metastatic testicular cancer. Lab Med. 2010;41:93–5.

Firmesse O, Alvaro E, Mogenet A, et al. Fate and effects of Camembert cheese micro-organisms in the human colonic microbiota of healthy volunteers after regular Camembert consumption. Int J Food Microbiol. 2008;125:176–81.

Fisher K, Phillips C. The ecology, epidemiology and virulence of *Enterococcus*. Microbiology. 2009;155:1749–57.

Flaherty JD, Levett PN, Dewhirst FE, Troe TE, Warren JR, Johnson S. Fatal case of endocarditis due to *Weissella confusa*. J Clin Microbiol. 2003;41:2237–9.

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science. 1995;269:496–512.

Florescu D, Hill L, Sudan D, Iwen PC. *Leuconostoc* bacteremia in pediatric patients with short bowel syndrome: case series and review. Pediatr Infect Dis J. 2008;27:1013–9.

Fodor AA, DeSantis TZ, Wylie KM, et al. The most wanted taxa from the human microbiome for whole genome sequencing. PLoS ONE. 2012;7:e41294.

Foligné B, Daniel C, Pot B. Probiotics from research to market: the possibilities, risks and challenges. Curr Opin Microbiol. 2013;16:284–92.

Forslund K, Sunagawa S, Kultima JR, Mende DR, Arumugam M, Typas A, Bork P. Country-specific antibiotic use practices impact the human gut resistome. Genome Res. 2013;23:1163–9.

Foxman EF, Iwasaki A. Genome-virome interactions: examining the role of common viral infections in complex disease. Nat Rev Microbiol. 2011;9:254–64.

Francavilla R, Miniello V, Magistà AM, et al. A randomized controlled trial of *Lactobacillus* GG in children with functional abdominal pain. Pediatrics. 2010;126:e1445–52.

Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A. 2007;104:13780–13785.

Franz CMAP, Huch M, Abriouel H, Holzapfel W, Gálvez A. Enterococci as probiotics and their implications in food safety. Int J Food Microbiol. 2011;151:125–40.

Gao XW, Mubasher M, Fang CY, Reifer C, Miller LE. Dose-response efficacy of a proprietary probiotic formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for

antibiotic-associated diarrhea and *Clostridium difficile*-associated diarrhea prophylaxis in adult patients. Am J Gastroenterol. 2010;105:1636–41.
Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312:1355–9.
Gilmore MS, Lebreton F, van Schaik W. Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. Curr Opin Microbiol. 2013;16:10–6.
Golińska E. Virulence factors of *Enterococcus* strains isolated from patients with inflammatory bowel disease. World J Gastroenterol. 2013;19:3562.
Gorkiewicz G, Thallinger GG, Trajanoski S, Lackner S, Stocker G, Hinterleitner T, Gülly C, Högenauer C. Alterations in the colonic microbiota in response to osmotic diarrhea. PLoS ONE. 2013;8:e55817.
Gosalbes MJ, Durbán A, Pignatelli M, Abellan JJ, Jiménez-Hernández N, Pérez-Cobas AE, Latorre A, Moya A. Metatranscriptomic approach to analyze the functional human gut microbiota. PLoS ONE. 2011;6:e17447.
Graessler J, Qin Y, Zhong H, et al. Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. Pharmacogenomics J. 2012. doi:10.1038/tpj.2012.43.
Grider JR, Piland BE. The peristaltic reflex induced by short-chain fatty acids is mediated by sequential release of 5-HT and neuronal CGRP but not BDNF. Am J Physiol Gastrointest Liver Physiol. 2007;292:G429–37.
Hamad I, Sokhna C, Raoult D, Bittar F. Molecular detection of eukaryotes in a single human stool sample from Senegal. PLoS ONE. 2012;7:e40888.
Hammerum AM. Enterococci of animal origin and their significance for public health: enterococci of animal origin and their significance. Clin Microbiol Infect. 2012;18:619–25.
Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol. 1998;5:R245–9.
Hansen EE, Lozupone CA, Rey FE, et al. Pan-genome of the dominant human gut-associated archaeon, *Methanobrevibacter smithii*, studied in twins. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4599–4606.
Hao WL, Lee YK. Microflora of the gastrointestinal tract: a review. Methods Mol Biol Clifton NJ. 2004;268:491–502.
Harlan NP, Kempker RR, Parekh SM, Burd EM, Kuhar DT. *Weissella confusa* bacteremia in a liver transplant patient with hepatic artery thrombosis. Transpl Infect Dis Off J Transplant Soc. 2011;13:290–3.
Hawrelak JA, Myers SP. The causes of intestinal dysbiosis: a review. Altern Med Rev J Clin Ther. 2004;9:180–97.
Hehemann J-H, Correc G, Barbeyron T, Helbert W, Czjzek M, Michel G. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature. 2010;464:908–12.
Heilig HGHJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, de Vos WM. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol. 2002;68:114–23.
Hemmerling A, Harrison W, Schroeder A, Park J, Korn A, Shiboski S, Foster-Rosales A, Cohen CR. Phase 2a study assessing colonization efficiency, safety, and acceptability of *Lactobacillus crispatus* CTV-05 in women with bacterial vaginosis. Sex Transm Dis. 2010;37:745–50.
Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, Lewis JD, Bushman FD. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. PLoS ONE. 2013;8:e66019.

Hong P-Y, Croix JA, Greenberg E, Gaskins HR, Mackie RI. Pyrosequencing-based analysis of the mucosal microbiota in healthy individuals reveals ubiquitous bacterial groups and micro-heterogeneity. PLoS ONE. 2011;6:e25042.

Hopkins MJ, Sharp R, Macfarlane GT. Variation in human intestinal microbiota with age. Dig Liver Dis Off J Ital Soc Gastroenterol Ital Assoc Study Liver 2002;34 Suppl 2:S12–18.

Hopkins MJ, Macfarlane GT, Furrie E, Fite A, Macfarlane S. Characterisation of intestinal bacteria in infant stools using real-time PCR and northern hybridisation analyses. FEMS Microbiol Ecol. 2005;54:77–85.

Howarth GS, Wang H. Role of endogenous microbiota, probiotics and their biological products in human health. Nutrients. 2013;5:58–81.

Hugon P, Lagier J-C, Robert C, Lepolard C, Papazian L, Musso D, Vialettes B, Raoult D. Molecular studies neglect apparently gram-negative populations in the human gut microbiota. J Clin Microbiol. 2013;51:3286–93.

Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486:207–14.

Hunt KM, Foster JA, Forney LJ, Schütte UME, Beck DL, Abdo Z, Fox LK, Williams JE, McGuire MK, McGuire MA. Characterization of the diversity and temporal stability of bacterial communities in human milk. PLoS ONE. 2011;6:e21313.

Huys G, Leisner J, Björkroth J. The lesser LAB gods. In: Von Wright A, editors. Lactic acid bacteria. CRC Press: Florida; 2011. p. 93–121.

Ishiyama K, Yamazaki H, Senda Y, Yamauchi H, Nakao S. *Leuconostoc* bacteremia in three patients with malignancies. J Infect Chemother Off J Jpn Soc Chemother. 2011;17:412–8.

Iwen PC, Mindru C, Kalil AC, Florescu DF. *Pediococcus acidilactici* endocarditis successfully treated with daptomycin. J Clin Microbiol. 2011;50:1106–8.

Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS ONE. 2010;5:e9836.

Janow G, Lambert B, Scheiner M, Rosen O, Goldman D, Soghier L. *Leuconostoc* septicemia in a preterm neonate on vancomycin therapy: case report and literature review. Am J Perinatol. 2008;26:089–91.

Jeffery IB, Claesson MJ, O'Toole PW, Shanahan F. Categorization of the gut microbiota: enterotypes or gradients? Nat Rev Microbiol. 2012;10:591–2.

Jofré ML, Sakurada ZA, Ulloa FMT, Hormázabal OJC, Godoy MV, Fernández OJ, Gutiérrez MM, Monteverde OMP, Castillo GM, Canales PA. *Leuconostoc* infections in patients with short gut syndrome, parenteral nutrition and continuous enteral feeding. Rev Chil Infectología Órgano Of Soc Chil Infectología. 2006;23:340–5.

José Pozuelo De Felipe M, García-Albiach R, Montesi Libois A, Rodríguez Borrajo C, Del Campo R, Rotger R. Monitoring of the succession of bacterial populations in infant faeces by PCR-denaturing gradient gel electrophoresis. Microb Ecol Heal Dis. 2005;17:205–211.

Kaeberlein T, Lewis K, Epstein SS. Isolating uncultivable microorganisms in pure culture in a simulated natural environment. Science. 2002;296:1127–9.

Kandler O. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek. 1983;49:209–24.

Kang BS, Seo JG, Lee GS, et al. Antimicrobial activity of enterocins from *Enterococcus faecalis* SL-5 against *Propionibacterium acnes*, the causative agent in acne vulgaris, and its therapeutic effect. J Microbiol Seoul Korea. 2009;47:101–9.

Karlsson FH, Tremaroli V, Nookaew I, Bergström G, Behre CJ, Fagerberg B, Nielsen J, Bäckhed F. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013;498:99–103.

Kelesidis T, Pothoulakis C. Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. Ther Adv Gastroenterol. 2012;5:111–25.

Keller MK, Twetman S. Acid production in dental plaque after exposure to probiotic bacteria. BMC Oral Heal. 2012;12:44.

Kerckhoffs APM, Samsom M, van Berge Henegouwen GP, Akkermans LMA, Nieuwenhuijs VB, Visser MR. Sampling microbiota in the human gastrointestinal tract. In: Gastrointestinal Microbiology. CRC Press: New York; 2006. p. 25–50.

Khoruts A, Dicksved J, Jansson JK, Sadowsky MJ. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent *Clostridium difficile*-associated diarrhea. J Clin Gastroenterol. 2010;44:354–60.

Kirtzalidou EI, Mitsou EK, Pramateftaki P, Kyriacou A. Screening fecal enterococci from Greek healthy infants for susceptibility to antimicrobial agents. Microb Drug Resist Larchmt N. 2012;18:578–85.

Ko SJ, Ryu B, Kim J, Hong BG, Yeo I, Lee BJ, Lee JM, Park JW. Effect of herbal extract granules combined with probiotic mixture on irritable bowel syndrome with diarrhea: study protocol for a randomized controlled trial. Trials. 2011;12:219.

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4578–4585.

Kolling GL, Wu M, Warren CA, Durmaz E, Klaenhammer TR, Guerrant RL. Lactic acid production by *Streptococcus thermophilus* alters *Clostridium difficile* infection and *in vitro* Toxin A production. Gut Microbes. 2012;3:523–9.

Koren O, Spor A, Felin J, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4592–4598.

Koren O, Goodrich JK, Cullender TC, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell. 2012;150:470–80.

Koren O, Knights D, Gonzalez A, Waldron L, Segata N, Knight R, Huttenhower C, Ley RE. A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets. PLoS Comput Biol. 2013;9:e1002863.

Kotzampassi K, Giamarellos-Bourboulis EJ, Voudouris A, Kazamias P, Eleftheriadis E. Benefits of a synbiotic formula (Synbiotic 2000Forte) in critically Ill trauma patients: early results of a randomized controlled trial. World J Surg. 2006;30:1848–55.

Koyama T, Kirjavainen PV, Fisher C, Anukam K, Summers K, Hekmat S, Reid G. Development and pilot evaluation of a novel probiotic mixture for the management of seasonal allergic rhinitis. Can J Microbiol. 2010;56:730–8.

Kumar A, Augustine D, Sudhindran S, Kurian AM, Dinesh KR, Karim S, Philip R. *Weissella confusa*: a rare cause of vancomycin-resistant gram-positive bacteraemia. J Med Microbiol. 2011;60:1539–41.

Lagier J-C, Million M, Hugon P, Armougom F, Raoult D. Human gut microbiota: repertoire and variations. Front Cell Infect Microbiol. 2012;2:136.

Lahtinen SJ, Tammela L, Korpela J, Parhiala R, Ahokoski H, Mykkänen H, Salminen SJ. Probiotics modulate the *Bifidobacterium* microbiota of elderly nursing home residents. Age. 2008;31:59–66.

LaTuga MS, Ellis JC, Cotton CM, Goldberg RN, Wynn JL, Jackson RB, Seed PC. Beyond bacteria: A study of the enteric microbial consortium in extremely low birth weight infants. PLoS ONE. 2011;6:e27858.

Lauková A. Potential applications of probiotic, bacteriocin-producing enterococci and their bacteriocins. In: Von Wright A, editors. Lactic acid bacteria. CRC Press: Florida; 2011. p. 39–61.

Le Gall G, Noor SO, Ridgway K, Scovell L, Jamieson C, Johnson IT, Colquhoun IJ, Kemsley EK, Narbad A. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. J Proteome Res. 2011;10:4208–18.

Leber B, Tripolt NJ, Blattl D, Eder M, Wascher TC, Pieber TR, Stauber R, Sourij H, Oettl K, Stadlbauer V. The influence of probiotic supplementation on gut permeability in patients with metabolic syndrome: an open label, randomized pilot study. Eur J Clin Nutr. 2012;66:1110–5.

Lee MR, Huang YT, Liao CH, Lai CC, Lee PI, Hsueh PR. Bacteraemia caused by *Weissella confusa* at a university hospital in Taiwan, 1997–2007. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2011;17:1226–31.

Leisner JJ, Laursen BG, Prévost H, Drider D, Dalgaard P. *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol Rev. 2007;31:592–613.

Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. 2006;124:837–48.

Leyer GJ, Li S, Mubasher ME, Reifer C, Ouwehand AC. Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics. 2009;124:e172–9.

Li WK, Chen YS, Wann SR, Liu YC, Tsai HC. *Lactococcus garvieae* endocarditis with initial presentation of acute cerebral infarction in a healthy immunocompetent man. Intern Med Tokyo Jpn. 2008;47:1143–6.

Lieske JC, Tremaine WJ, De Simone C, O'Connor HM, Li X, Bergstralh EJ, Goldfarb DS. Diet, but not oral probiotics, effectively reduces urinary oxalate excretion and calcium oxalate supersaturation. Kidney Int. 2010;78:1178–85.

Ligaarden SC, Axelsson L, Naterstad K, Lydersen S, Farup PG. A candidate probiotic with unfavourable effects in subjects with irritable bowel syndrome: a randomised controlled trial. BMC Gastroenterol. 2010;10:16.

Liu J, Wu D, Ahmed A, Li X, Ma Y, Tang L, Mo D, Ma Y, Xin Y. Comparison of the gut microbe profiles and numbers between patients with liver cirrhosis and healthy individuals. Curr Microbiol. 2012;65:7–13.

Maccaferri S, Biagi E, Brigidi P. Metagenomics: key to human gut microbiota. Dig Dis Basel Switz. 2011;29:525–30.

Macfarlane S, Dillon JF. Microbial biofilms in the human gastrointestinal tract. J Appl Microbiol. 2007;102:1187–96.

Magne F, Abély M, Boyer F, Morville P, Pochart P, Suau A. Low species diversity and high interindividual variability in faeces of preterm infants as revealed by sequences of 16S rRNA genes and PCR-temporal temperature gradient gel electrophoresis profiles. FEMS Microbiol Ecol. 2006;57:128–38.

Makarova KS, Koonin EV. Evolutionary genomics of lactic acid bacteria. J Bacteriol. 2006;189:1199–208.

Maldonado J, Lara-Villoslada F, Sierra S, Sempere L, Gómez M, Rodriguez JM, Boza J, Xaus J, Olivares M. Safety and tolerance of the human milk probiotic strain *Lactobacillus salivarius* CECT5713 in 6-month-old children. Nutr Burbank Los Angeles Cty Calif. 2010;26:1082–7.

Mangalat N, Liu Y, Fatheree NY, et al. Safety and tolerability of *Lactobacillus reuteri* DSM 17938 and effects on biomarkers in healthy adults: results from a randomized masked trial. PLoS ONE. 2012;7:e43910.

Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. Nat Rev Gastroenterol Hepatol. 2012;9:599–608.

Mantzourani M, Gilbert SC, Fenlon M, Beighton D. Non-oral *Bifidobacteria* and the aciduric microbiota of the denture plaque biofilm. Mol Oral Microbiol. 2010;25:190–9.

Marchesi JR. Prokaryotic and eukaryotic diversity of the human gut. Adv Appl Microbiol. 2010;72:43–62.

Markowitz VM, Chen I-MA, Chu K, et al. IMG/M-HMP: a metagenome comparative analysis system for the Human Microbiome Project. PLoS ONE. 2012;7:e40151.

Martens U, Enck P, Zieseniss E. Probiotic treatment of irritable bowel syndrome in children. Ger Med Sci GMS E-J. 2010;8:Doc07.

Martín R, Langa S, Reviriego C, Jimínez E, Marín ML, Xaus J, Fernández L, Rodríguez JM. Human milk is a source of lactic acid bacteria for the infant gut. J Pediatr. 2003;143:754–8.

Mastro TD, Spika JS, Lozano P, Appel J, Facklam RR. Vancomycin-resistant *Pediococcus acidilactici*: nine cases of bacteremia. J Infect Dis. 1990;161:956–60.

Maukonen J, Mättö J, Suihko M-L, Saarela M. Intra-individual diversity and similarity of salivary and faecal microbiota. J Med Microbiol. 2008;57:1560–8.

Maukonen J, Simões C, Saarela M. The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. FEMS Microbiol Ecol. 2012;79:697–708.

Maurice CF, Haiser HJ, Turnbaugh PJ. Xenobiotics shape the physiology and gene expression of the active human gut microbiome. Cell. 2013;152:39–50.

Ménard S, Candalh C, Bambou JC, Terpend K, Cerf-Bensussan N, Heyman M. Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. Gut. 2004;53:821–8.

Merenstein DJ, Foster J, D'Amico F. A randomized clinical trial measuring the influence of kefir on antibiotic-associated diarrhea: the measuring the influence of Kefir (MILK) Study. Arch Pediatr Adolesc Med. 2009;163:750–4.

Miller TL, Wolin MJ, Conway de Macario E, Macario AJ. Isolation of *Methanobrevibacter smithii* from human feces. Appl Environ Microbiol. 1982;43:227–232.

Miller TL, Wolin MJ. *Methanosphaera stadtmaniae* gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. Arch Microbiol. 1985;141:116–22.

Million M, Maraninchi M, Henry M, Armougom F, Richet H, Carrieri P, Valero R, Raccah D, Vialettes B, Raoult D. Obesity-associated gut microbiota is enriched in *Lactobacillus reuteri* and depleted in *Bifidobacterium animalis* and *Methanobrevibacter smithii*. Int J Obes. 2005;36:817–825.

Minot S, Sinha R, Chen J, Li H, Keilbaugh SA, Wu GD, Lewis JD, Bushman FD. The human gut virome: inter-individual variation and dynamic response to diet. Genome Res. 2011;21:1616–25.

Minot S, Bryson A, Chehoud C, Wu GD, Lewis JD, Bushman FD. Rapid evolution of the human gut virome. Proc Natl Acad Sci U S A. 2013;110:12450–5.

Monsen T, Granlund M, Olofsson K, Olsen B. *Leuconostoc* spp. septicaemia in a child with short bowel syndrome. Scand J Infect Dis. 1997;29:310–1.

Moore WE, Moore LH. Intestinal floras of populations that have a high risk of colon cancer. Appl Environ Microbiol. 1995;61:3202–7.

Morrow LE, Kollef MH, Casale TB. Probiotic prophylaxis of ventilator-associated pneumonia: a blinded, randomized, controlled trial. Am J Respir Crit Care Med. 2010;182:1058–64.

Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, Fontana L, Henrissat B, Knight R, Gordon JI. Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science. 2011;332:970–4.

Mueller S, Saunier K, Hanisch C, et al. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol. 2006;72:1027–33.

Murray BE. The life and times of the *Enterococcus*. Clin Microbiol Rev. 1990;3:46–65.

Nakamura N, Lin HC, McSweeney CS, Mackie RI, Gaskins HR. Mechanisms of microbial hydrogen disposal in the human colon and implications for health and disease. Annu Rev Food Sci Technol. 2010;1:363–95.

Nam Y-D, Chang H-W, Kim K-H, Roh SW, Kim M-S, Jung M-J, Lee S-W, Kim J-Y, Yoon J-H, Bae J-W. Bacterial, archaeal, and eukaryal diversity in the intestines of Korean people. J Microbiol Seoul Korea. 2008;46:491–501.

Nelson KE, Weinstock GM, et al. Human Microbiome Jumpstart Reference Strains Consortium. A catalog of reference genomes from the human microbiome. Science. 2010;328:994–999.

Nermes M, Kantele JM, Atosuo TJ, Salminen S, Isolauri E. Interaction of orally administered *Lactobacillus rhamnosus* GG with skin and gut microbiota and humoral immunity in infants with atopic dermatitis. Clin Exp Allergy J Br Soc Allergy Clin Immunol. 2011;41:370–7.

Ngugi BM, Hemmerling A, Bukusi EA, Kikuvi G, Gikunju J, Shiboski S, Fredricks DN, Cohen CR. Effects of bacterial vaginosis-associated bacteria and sexual intercourse on vaginal colonization with the probiotic *Lactobacillus crispatus* CTV-05. Sex Transm Dis. 2011;38:1020–7.

Nielsen DS, Moller PL, Rosenfeldt V, Paerregaard A, Michaelsen KF, Jakobsen M. Case study of the distribution of mucosa-associated *Bifidobacterium* species, *Lactobacillus* species, and other lactic acid bacteria in the human colon. Appl Environ Microbiol. 2003;69:7545–8.

Niers L, Martín R, Rijkers G, Sengers F, Timmerman H, van Uden N, Smidt H, Kimpen J, Hoekstra M. The effects of selected probiotic strains on the development of eczema (the PandA study). Allergy. 2009;64:1349–58.

Nishimura I, Igarashi T, Enomoto T, Dake Y, Okuno Y, Obata A. Clinical efficacy of halophilic lactic acid bacterium *Tetragenococcus halophilus* Th221 from soy sauce moromi for perennial allergic rhinitis. Allergol Int. 2009;58:179–85.

Nomura T, Tsuchiya Y, Nashimoto A, Yabusaki H, Takii Y, Nakagawa S, Sato N, Kanbayashi C, Tanaka O. Probiotics reduce infectious complications after pancreaticoduodenectomy. Hepatogastroenterology. 2007;54:661–3.

O'Shea EF, Gardiner GE, O'Connor PM, Mills S, Ross RP, Hill C. Characterization of enterocin- and salivaricin-producing lactic acid bacteria from the mammalian gastrointestinal tract. FEMS Microbiol Lett. 2009;291:24–34.

Ogilvie LA, Firouzmand S, Jones BV. Evolutionary, ecological and biotechnological perspectives on plasmids resident in the human gut mobile metagenome. Bioeng Bugs. 2012;3:13–31.

Ogilvie LA, Bowler LD, Caplin J, Dedi C, Diston D, Cheek E, Taylor H, Ebdon JE, Jones BV. Genome signature-based dissection of human gut metagenomes to extract subliminal viral sequences. Nat Commun. 2013;4:2420.

Olano A, Chua J, Schroeder S, Minari A, La Salvia M, Hall G. *Weissella confusa* (basonym: *Lactobacillus confusus*) bacteremia: a case report. J Clin Microbiol. 2001;39:1604–7.

Ott SJ, Kühbacher T, Musfeldt M, Rosenstiel P, Hellmig S, Rehman A, Drews O, Weichert W, Timmis KN, Schreiber S. Fungi and inflammatory bowel diseases: alterations of composition and diversity. Scand J Gastroenterol. 2008;43:831–41.

Oxley APA, Lanfranconi MP, Würdemann D, Ott S, Schreiber S, McGenity TJ, Timmis KN, Nogales B. Halophilic archaea in the human intestinal mucosa. Environ Microbiol. 2010;12:2398–410.

Parfrey LW, Walters WA, Knight R. Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. Front Microbiol. 2011;2:153.

Park SK, Park DI, Choi JS, Kang MS, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI. The effect of probiotics on *Helicobacter pylori* eradication. Hepatogastroenterology. 2007;54:2032–6.

Patil DP, Dhotre DP, Chavan SG, et al. Molecular analysis of gut microbiota in obesity among Indian individuals. J Biosci. 2012;37:647–57.

Peris-Bondia F, Latorre A, Artacho A, Moya A, D'Auria G. The active human gut microbiota differs from the total microbiota. PLoS ONE. 2011;6:e22448.

Pfleiderer A, Lagier J-C, Armougom F, Robert C, Vialettes B, Raoult D. Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol. 2013;32:1471–81.

Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. Br J Nutr. 2013:1–16.

Prakash VP, Rao SR, Parija SC. Emergence of unusual species of enterococci causing infections, South India. BMC Infect Dis. 2005;5:14.

Prakash S, Malgorzata Urbanska A. Colon-targeted delivery of live bacterial cell biotherapeutics including microencapsulated live bacterial cells. Biol Targets Ther. 2008;2:355–78.

Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464:59–65.

Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490:55–60.

Rajilić-Stojanović M, Heilig HGHJ, Tims S, Zoetendal EG, de Vos WM. Long-term monitoring of the human intestinal microbiota composition: long-term monitoring of the human intestinal microbiota. Environ Microbiol. 2013;15:1146–59.

Raman M, Ahmed I, Gillevet PM, et al. Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol Off Clin Pr J Am Gastroenterol Assoc. 2013;11(868–875):e1–3.

Resta-Lenert S, Barrett KE. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). Gut. 2003;52:988–97.

Reuter G. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol. 2001;2:43–53.

Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, Gordon JI. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature. 2010;466:334–8.

Rieu-Lesme F, Delbès C, Sollelis L. Recovery of partial 16S rDNA sequences suggests the presence of *Crenarchaeota* in the human digestive ecosystem. Curr Microbiol. 2005;51:317–21.

Riezzo G, Orlando A, D'Attoma B, Guerra V, Valerio F, Lavermicocca P, De Candia S, Russo F. Randomised clinical trial: efficacy of Lactobacillus paracasei-enriched artichokes in the treatment of patients with functional constipation—a double-blind, controlled, crossover study. Aliment Pharmacol Ther. 2012;35:441–50.

Ringel-Kulka T, Palsson OS, Maier D, Carroll I, Galanko JA, Leyer G, Ringel Y. Probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 versus placebo for the symptoms of bloating in patients with functional bowel disorders: a double-blind study. J Clin Gastroenterol. 2011;45:518–25.

Robles Alonso V, Guarner F. Linking the gut microbiota to human health. Br J Nutr 2013;109 Suppl 2:S21–26.

Roccarina D, Lauritano EC, Gabrielli M, Franceschi F, Ojetti V, Gasbarrini A. The role of methane in intestinal diseases. Am J Gastroenterol. 2010;105:1250–6.

Roediger WE. Anaerobic bacteria, the colon and colitis. Aust N Z J Surg. 1980;50:73–5.

Rose MA, Stieglitz F, Köksal A, Schubert R, Schulze J, Zielen S. Efficacy of probiotic *Lactobacillus* GG on allergic sensitization and asthma in infants at risk. Clin Exp Allergy J Br Soc Allergy Clin Immunol. 2010;40:1398–405.

Russo G, Iannetta M, D'Abramo A, et al. *Lactococcus garvieae* endocarditis in a patient with colonic diverticulosis: first case report in Italy and review of the literature. New Microbiol. 2012;35:495–501.

Sakata H, Yoshioka H, Fujita K. Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. Eur J Pediatr. 1985;144:186–90.

Salimnia H, Alangaden GJ, Bharadwaj R, Painter TM, Chandrasekar PH, Fairfax MR. *Weissella confusa*: an unexpected cause of vancomycin-resistant gram-positive bacteremia in immunocompromised hosts. Transpl Infect Dis Off J Transplant Soc. 2011;13:294–8.

Samuel BS, Hansen EE, Manchester JK, Coutinho PM, Henrissat B, Fulton R, Latreille P, Kim K, Wilson RK, Gordon JI. Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. Proc Natl Acad Sci U S A. 2007;104:10643–8.

Sanchez E, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y. Intestinal *Bacteroides* species associated with coeliac disease. J Clin Pathol. 2010;63:1105–11.

Sarma PS, Mohanty S. *Pediococcus acidilactici* pneumonitis and bacteremia in a pregnant woman. J Clin Microbiol. 1998;36:2392–3.

Savard P, Lamarche B, Paradis M-E, Thiboutot H, Laurin É, Roy D. Impact of *Bifidobacterium animalis* subsp. *lactis* BB-12 and, *Lactobacillus acidophilus* LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. Int J Food Microbiol. 2011;149:50–7.

Scanlan PD, Marchesi JR. Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and-independent analysis of faeces. ISME J. 2008;2:1183–93.

Scanlan PD, Shanahan F, Marchesi JR. Human methanogen diversity and incidence in healthy and diseased colonic groups using mcrA gene analysis. BMC Microbiol. 2008;8:79.

Scher JU, Sczesnak A, Longman RS, et al (2013) Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. eLife 2:e01202–e01202.

Schleifer KH, Kilpper-Balz R. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the Genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int J Syst Bacteriol. 1984;34:31–4.

Schulze J, Sonnenborn U. Yeasts in the gut: from commensals to infectious agents. Dtsch Ärzteblatt Int. 2009;106:837–42.

Schwartz S, Friedberg I, Ivanov IV, Davidson LA, Goldsby JS, Dahl DB, Herman D, Wang M, Donovan SM, Chapkin RS. A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in immune response. Genome Biol. 2012;13:r32.

Sela DA, Chapman J, Adeuya A, et al. The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A. 2008;105:18964–9.

Shin JH, Kim DI, Kim HR, Kim DS, Kook J-K, Lee JN. Severe infective endocarditis of native valves caused by *Weissella confusa* detected incidentally on echocardiography. J Infect. 2007;54:e149–51.

Shin J, Her M, Moon C, Kim D, Lee S, Jung S. *Leuconostoc* bacteremia in a patient with amyloidosis secondary to rheumatoid arthritis and tuberculosis arthritis. Mod Rheumatol. 2011;21:691–5.

Siebrasse EA, Reyes A, Lim ES, Zhao G, Mkakosya RS, Manary MJ, Gordon JI, Wang D. Identification of MW polyomavirus, a novel polyomavirus in human stool. J Virol. 2012;86:10321–6.

Sim JS, Kim HS, Oh KJ, Park MS, Jung EJ, Jung YJ, Kang DG, Seo SI, Kim WJ, Jang MK. Spontaneous bacterial peritonitis with sepsis caused by *Enterococcus hirae*. J Korean Med Sci. 2012;27:1598–600.

Sire JM, Donnio PY, Mesnard R, Pouëdras P, Avril JL. Septicemia and hepatic abscess caused by *Pediococcus acidilactici*. Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol. 1992;11:623–5.

Smith MI, Yatsunenko T, Manary MJ, et al. Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. Science. 2013;339:548–54.

Solís G, de los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M. Establishment and development of lactic acid bacteria and *Bifidobacteria* microbiota in breast-milk and the infant gut. Anaerobe. 2010;16:307–310.

Sommer F, Bäckhed F. The gut microbiota—masters of host development and physiology. Nat Rev Microbiol. 2013;11:227–38.

Stapleton AE, Au-Yeung M, Hooton TM, Fredricks DN, Roberts PL, Czaja CA, Yarova-Yarovaya Y, Fiedler T, Cox M, Stamm WE. Randomized, placebo-controlled phase 2 trial of a *Lactobacillus crispatus* probiotic given intravaginally for prevention of recurrent urinary tract infection. Clin Infect Dis Off Publ Infect Dis Soc Am. 2011;52:1212–7.

Stecher B, Maier L, Hardt W-D. Blooming in the gut: how dysbiosis might contribute to pathogen evolution. Nat Rev Microbiol. 2013;11:277–84.

Stern A, Mick E, Tirosh I, Sagy O, Sorek R. CRISPR targeting reveals a reservoir of common phages associated with the human gut microbiome. Genome Res. 2012;22:1985–94.

Suh B. Resolution of persistent *Pediococcus* bacteremia with daptomycin treatment: case report and review of the literature. Diagn Microbiol Infect Dis. 2010;66:111–5.

Surono IS, Koestomo FP, Novitasari N, Zakaria FR, Yulianasari, Koesnandar. Novel probiotic *Enterococcus faecium* IS-27526 supplementation increased total salivary sIgA level and bodyweight of pre-school children: a pilot study. Anaerobe. 2011;17:496–500.

Szajewska H, Gyrczuk E, Horvath A. *Lactobacillus reuteri* DSM 17938 for the management of infantile colic in breastfed infants: a randomized, double-blind, placebo-controlled trial. J Pediatr. 2013;162:257–62.

Tagg J, Wescombe P, Burton J. *Streptococcus*. In: Von Wright A, editor. Lactic acid bacteria. CRC Press: Florida; 2011. p. 123–146.

Taur Y, Xavier JB, Lipuma L, et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012;55:905–14.

Teixeira LM, Carvalho MG, Merquior VL, Steigerwalt AG, Brenner DJ, Facklam RR. Phenotypic and genotypic characterization of *Vagococcus fluvialis*, including strains isolated from human sources. J Clin Microbiol. 1997;35:2778–81.

Tillisch K, Labus J, Kilpatrick L, et al. Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology. 2013;144(1394–1401):e4.

Trautvetter U, Ditscheid B, Kiehntopf M, Jahreis G. A combination of calcium phosphate and probiotics beneficially influences intestinal *Lactobacilli* and cholesterol metabolism in humans. Clin Nutr Edinb Scotl. 2012;31:230–7.

Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457:480–4.

Turroni F, Foroni E, Pizzetti P, et al. Exploring the diversity of the *Bifidobacterial* population in the human intestinal tract. Appl Environ Microbiol. 2009;75:1534–45.

Turroni F, Peano C, Pass DA, et al. Diversity of *Bifidobacteria* within the infant gut microbiota. PLoS ONE. 2012;7:e36957.

Tursi A, Brandimarte G, Elisei W, et al. Randomised clinical trial: mesalazine and/or probiotics in maintaining remission of symptomatic uncomplicated diverticular disease—a double-blind, randomised, placebo-controlled study. Aliment Pharmacol Ther. 2013;38:741–51.

Tyakht AV, Kostryukova ES, Popenko AS, et al. Human gut microbiota community structures in urban and rural populations in Russia. Nat Commun. 2013. doi:10.1038/ncomms3469.

Van Puyenbroeck K, Hens N, Coenen S, Michiels B, Beunckens C, Molenberghs G, Van Royen P, Verhoeven V. Efficacy of daily intake of *Lactobacillus casei* Shirota on respiratory symptoms and influenza vaccination immune response: a randomized, double-blind, placebo-controlled trial in healthy elderly nursing home residents. Am J Clin Nutr. 2012;95:1165–71.

Vanhoutte T, Huys G, Brandt E, Swings J. Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. FEMS Microbiol Ecol. 2004;48:437–46.

Verberkmoes NC, Russell AL, Shah M, et al. Shotgun metaproteomics of the human distal gut microbiota. ISME J. 2009;3:179–89.

Vrieze A, Van Nood E, Holleman F, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology. 2012;143(913–916):e7.

Walter J. Ecological role of *Lactobacilli* in the gastrointestinal tract: implications for fundamental and biomedical research. Appl Environ Microbiol. 2008;74:4985–96.

Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol. 2001;67:2578–85.

Wang M, Ahrné S, Jeppsson B, Molin G. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. FEMS Microbiol Ecol. 2005;54:219–31.

Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472:57–63.

Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, Jia W, Cai S, Zhao L. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. ISME J. 2012;6:320–9.

Wang L, Zhang J, Guo Z, Kwok L, Ma C, Zhang W, Lv Q, Huang W, Zhang H. The impact of oral consumption of the probiotic *Lactobacillus plantarum* P-8 on the faecal microbiota, SIgA, SCFAs and TBAs of subjects of different age. Nutrition. 2013. doi:10.1016/j.nut.2013.11.018.

Wanke M, Szajewska H. Lack of an effect of *Lactobacillus reuteri* DSM 17938 in preventing nosocomial diarrhea in children: a randomized, double-blind, placebo-controlled trial. J Pediatr. 2012;161(40–43):e1.

Ward TL, Hosid S, Ioshikhes I, Altosaar I. Human milk metagenome: a functional capacity analysis. BMC Microbiol. 2013;13:116.

Wassenberg J, Nutten S, Audran R, Barbier N, Aubert V, Moulin J, Mercenier A, Spertini F. Effect of *Lactobacillus paracasei* ST11 on a nasal provocation test with grass pollen in allergic rhinitis. Clin Exp Allergy J Br Soc Allergy Clin Immunol. 2011;41:565–73.

Wescombe PA, Hale JD, Heng NC, Tagg JR. Developing oral probiotics from *Streptococcus salivarius*. Future Microbiol. 2012;7:1355–71.

Westerbeek EAM, van den Berg A, Lafeber HN, Knol J, Fetter WPF, van Elburg RM. The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr Edinb Scotl. 2006;25:361–8.

Willing BP, Dicksved J, Halfvarson J, Andersson AF, Lucio M, Zheng Z, Järnerot G, Tysk C, Jansson JK, Engstrand L. A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. Gastroenterology. 2010;139(1844–1854):e1.

Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334:105–8.

Wylie KM, Weinstock GM, Storch GA. Emerging view of the human virome. Transl Res J Lab Clin Med. 2012;160:283–90.

Xu J, Yang H, Lai X, Fu X, Wu J, Huang L, Yu X, Wu Y, Wu Y, Liu B. Etiological study for a case of multi-bacterial synergistic gangrene. Chin Sci Bull. 1997;42:511–7.

Yang R, Argimon S, Li Y, Gu H, Zhou X, Caufield PW. Determining the genetic diversity of *Lactobacilli* from the oral cavity. J Microbiol Methods. 2010;82:163–9.

Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486:222–7.

Yossuck P, Miller-Canfield P, Moffett K, Graeber J. *Leuconostoc* spp sepsis in an extremely low birth weight infant: a case report and review of the literature. W V Med J. 2009;105:24–7.

Zhang G, Chen R, Rudney JD. Streptococcus cristatus attenuates *Fusobacterium nucleatum*-induced interleukin-8 expression in oral epithelial cells. J Periodontal Res. 2008;43:408–16.

Zhang H, DiBaise JK, Zuccolo A, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A. 2009;106:2365–70.

Zhang L, Kinkelaar D, Huang Y, Li Y, Li X, Wang HH. Acquired antibiotic resistance: are we born with it? Appl Environ Microbiol. 2011;77:7134–41.

Zivkovic AM, German JB, Lebrilla CB, Mills DA. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4653–4658.

Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ, Bork P, Wels M, de Vos WM, Kleerebezem M. The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME J. 2012;6:1415–26.

Zupancic ML, Cantarel BL, Liu Z, et al. Analysis of the gut microbiota in the old order Amish and its relation to the metabolic syndrome. PLoS ONE. 2012;7:e43052.

Chapter 7
Application of Lactic Acid Bacteria for Animal Production

Yimin Cai, Huili Pang, Zhongfang Tan, Yanping Wang, Jianguo Zhang, Chuncheng Xu, Jinsong Yang and Yang Cao

Abstract The effect of lactic acid bacteria (LAB) on silage storage and livestock production has caught the increasing attention of many scientists. The exploration of LAB with a high-performance in silage making is noteworthy for future research and is extremely important for developing safe livestock production and for increasing self-supplying feed. LAB not only influences the large intestine by affecting the intestinal flora but also by affecting other organs via the modulation of immunological parameters and intestinal permeability or via the production of bioactive or regulatory metabolites. This chapter first discusses the use of LAB in animal feeding. The protective effects of LAB that can inhibit food-borne

Y. Cai (✉)
Japan International Research Center for Agricultural Sciences, Tsukuba, Japan
e-mail: cai@affrc.go.jp

H. Pang · Z. Tan · Y. Wang
Zhengzhou University, Zhengzhou, China
e-mail: pang@zzu.edu.cn

Z. Tan
e-mail: tzhongfang@zzu.edu.cn

Y. Wang
e-mail: wyp@zzu.edu.cn

J. Zhang
South China Agricultural University, Guangzhou, China
e-mail: zhangjg@scau.edu.cn

C. Xu
China Agricultural University, Beijing, China
e-mail: xucc@cau.edu.cn

J. Yang
Hainan University, Haikou, China
e-mail: food868@163.com

Y. Cao
Heilongjiang Bayi Agricultural University, Daqing, China
e-mail: hbdkcaoyang@163.com

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_7,
© Springer Science+Business Media Dordrecht 2014

pathogens in live animals before slaughter have been described. LAB are capable of producing inhibitory substances, other than organic acids, that have inhibitory activities against different microorganisms. However, not all LAB will reduce food-borne pathogens in farm animals; therefore, the careful selection of strains to be administered under appropriate conditions is important. In addition, effects of LAB on feed preparation and animal production are described and reviewed.

Keywords LAB · Animal production · Silage · Feed

7.1 Lactic Acid Bacteria and Animal Health

7.1.1 Lactic Acid Bacteria and Bifidobacteria in Animal Intestine

Animal intestines are colonised by many microorganisms, and these organisms differ in both type and number. Because many types and large quantities of microorganisms settle in the animal gut, intestinal microbial flora play an extremely important role in the physiology and health of the host. The correlation between these microorganisms and the host is not only positive (partial benefit symbiosis, Aioi and reciprocity phenomena) but also negative (competition and partial damage phenomena), and this correlation jointly maintains a micro-ecological balance. Among these microorganisms, lactic acid bacteria (LAB) and bifidobacteria, which are dominant intestinal flora, have received worldwide attention and examination. Studies have shown that LAB and bifidobacteria can regulate the common ratio of normal intestinal flora and maintain the intestinal balance of this ecosystem.

Many of the LAB species are significant constituents of the normal gut microbiota of humans and animals, and their occurrence and number are host-dependent. Lactobacilli have an inhibitory effect on pathogens. Of the bacteria in the gut, the most representative and undoubtedly beneficial bacteria are *Lactobacillus*, which include *L. acidophilus*, *L. coprophilus*, *L. intestinalis*, *L. lacis*, *L. curvatus* and *L. brevis*. Van Schie et al. reported that the lactic acid and volatile fatty acids that are produced by *Lactobacillus* play important roles in reducing the *Enterobacteriaceae*, such as *Salmonella*, that colonise the intestine.

The small intestine has the largest quantity of *Lactobacillus* in the gut and in the faeces within equal (10^5–10^8 cfu g^{-1}) content, whereas for pig and chicken, it is the rectum, followed by the jejunum. LAB in the digestive tract are primarily distributed in the lower part of the small intestine, inhibiting harmful bacteria from the mouth reproductive. *Lactobacillus* is Gram-positive, nonspore, slender and curved bacillus, sometimes as spherical or rod-like and arranged as bar or chain, no power and some with bipolar dyeing. It produces lactate under fermentation, is

catalase-negative, cannot gelatine liquefaction, reduces nitrate and produces pigment and its colonies are occasionally yellow or red. *Lactobacillus* is microaerophilic and can grow with 5–10 % CO_2, but it grows better in an anaerobic environment. The optimum growth temperature for *Lactobacillus* is 30–40 °C, with a pH range of 5.5–6.2; however, *Lactobacillus* also grows at pH 3.5. This complicated group can be divided into at least 18 genera, including more than 200 types. With few exceptions, most of these bacteria are essential to the animal body, have important physiological functions and are extensively present in the animal gut.

Enterococci are found naturally in food products. These commensal microorganisms are commonly found in humans and animals. *Enterococcus faecium* and *En. faecalis* are the most common Enterococci in the human gastrointestinal tract (GIT), whereas *En. faecium* is prevalent in animals (Biavati and Mattarelli 2006). *Lactococcus lactis* is not conducive to the colonisation of the digestive tracts of animals and, similar to neutral particles and liposomes, is only a transient through the intestinal tract.

In 1899, Diesel of the Pasteur Institute found many anaerobic LAB that had difficulty surviving in the air in the infant gut during a survey of intestinal bacteria in the faeces of breastfed infants and that most of the bacteria had Y or V bifurcation; thus, these anaerobic LAB were named *Bifidobacterium*. Thus far, at least 28 types of bifidobacteria have been isolated from human and animal (cattle, sheep, rabbits, rats, pigs, chickens and bees) intestines, cavities in human teeth, vaginas, wastewater, etc. In addition, only *Bifidobacterium dentium* is pathogenic; no other pathogenic bifidobacteria have been reported yet. To date, nine *Bifidobacterium* species have been found in the intestines, including *B. bifidum*, *B. longum*, *B. breve*, *B. infantis*, *B. adolescentis*, *B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum* and *B. dentium*.

The amounts and types of *Bifidobacterium* in the gut vary with age and the number of bacteria reaches the highest value within a few days after birth and then gradually decreases, whereas the number of other intestinal bacteria increases. *B. infantis*, *B. breve*, *B. bifidum* and *B. longum* are the main bacteria in infants; *B. longum* and *B. adolescentis* are dominant in adults; and in the elderly, *B. adolescentis*, *B. longum* and *B. bifidum* are the most important bacteria.

In the intestinal tracts of animal, bifidobacteria are considered one of the key genera. Their presence in high numbers is associated with the good health status of the host. There is a general belief that bifidobacteria are helpful in maintaining the appropriate balance of the microbiota in the GIT, which reduces the risk of pathogen infection. Several species of bifidobacteria are host-specific (Fisher and Phillips 2009).

Bifidobacterium is a genus of Gram-positive rod bacteria, which do not form spores, do not produce energy and have negative acid-fast staining. Additionally, polymorphic bifurcation or mycobacterium is the important nature of the genus. *Bifidobacterium*, which is the dominant intestinal flora, is important for maintaining a healthy body. Additionally, *Bifidobacterium* is the major component of the intestinal membrane bacteria group, which plays an important role in the

maintenance of normal bowel movements, while partially preventing the fixed planting of pathogenic bacteria. Meanwhile, bifidobacteria directly antagonise many pathogens both in vivo and in vitro. Moreover, bifidobacteria can produce extracellular glycosidase, which degrades complex polysaccharides on intestinal epithelial cells; these complex polysaccharides are receptors of potential pathogens and bacterial toxins. *Bifidobacterium* can synthesise vitamins, such as V_{B1}, V_{B2}, V_{B12}, niacin and folic acid and the acid environment is conducive to the absorption of divalent iron, V_D and calcium. *Bifidobacterium* has anti-tumour activity, which can degrade N-nitrosamines and other carcinogenic factors to prevent tumours and inhibit tumour growth by enhancing the body's nonspecific and specific immunoreaction.

Complex sugars (fibre or semifibre) that are present in plant cell walls cannot be used by nonruminants; in contrast, microbiota that lives in the rumen and reticulum of ruminants enable energy to be obtained from the fibre. In addition, nonprotein nitrogen cannot be utilised by nonruminants but can be used by rumen bacteria for protein synthesis, and bacterial protein that is synthesised in the rumen is the main source of amino acids that are required for ruminants. The rumen is a good natural environment for anaerobic microorganism reproduction and can accommodate many fermenters. Microbes play a dominant position in a series of complex digestion metabolic processes that are conducted in the rumen. The rumen provides rich food for microbial growth and reproduction. Food and water enter the rumen continually to supply required nutrients to microbes.

Rumen microorganisms play an important role in the diet degradation process, when there is a high proportion of concentrated feed. The rumen LAB include *Streptococcus bovis*, *S. equinus*, *Lactobacillus*, *Bacteroides ruminicola* and *Bifidobacterium*, of which *S. bovis* is the most common. It has been reported that the number of *S. bovis* is 10^4–10^7 cfu g^{-1} in rumen under entirely foraged diet conditions, but when the diet is primarily cereal-based, the number can increase to 10^{11} cfu g^{-1}, which can be attributed to the faster growth rate of streptococci in comparison with other rumen microbes. *Lactobacillus* is a type that is commonly found in the GIT of monogastric animals; however, its number in the rumen of ruminant animals is low.

7.1.2 Microbiota of Animal Gastrointestinal Tract and Rumen

Microbes are an important and indispensable component of the animal gut. The newborn animal digestive tract is sterile; a few hours later, with sucking, feeding and other processes that occur in the digestive tract, microorganisms, such as *Escherichia coli*, will inhabit the intestines and will coexist with the host for life (Champagne et al. 2005).

Intestinal microflora, which are highly complex and are composed of diverse groups, are an important part of the animal body. Animals are super organisms that are composed of 10 % body cells and 90 % microbial cells, and intestinal microorganisms are the largest and most complex microbial communities, which include more than 10^{14} and thousands of microbial cells, which are involved in digestion, nutrient absorption, energy production, fat metabolism, immune modulation, disease resistance and other aspects that affect the health of the body.

Comparing the distribution of flora, the dominant bacteria in the animal stomach are primarily acid-resistant and facultative anaerobes; bile salts in the duodenum limit the existence of bacteria; the number and types of bacteria in the jejunum and in the ileum gradually increase. Additionally, lactobacilli and bifidobacteria are the most dominant bacteria, followed by peptococci, enterococci, clostridium and staphylococci. The large intestine is the main place for gastrointestinal microbial activity, with the cecum and colon as the maximum position for bacterial colonisation, which primarily consists of anaerobic bacteria, such as *Bacteroides* and many Gram-positive bacteria. Strictly anaerobic bacteria include nonspore-forming bacteria, such as eubacterium, bifidobacteria, streptococci, peptostreptococci and peptococci and spore-forming bacteria, such as clostridium and Plectridiaceae. Facultative anaerobes are 10–100 times less common than anaerobes, which include streptococci and lactobacilli.

Bifidobacterium is a physiologically certain bacterium of humans and mammals, which is almost undetectable in the upper small intestine, whereas the number in the lower small intestine reaches 10^3–10^5 g^{-1} and reaches 10^3–10^{12} cfu g^{-1} in colon faeces. *Bifidobacterium* species have an indirectly cytotoxic effect, which not only prevents breeding of harmful bacteria in the intestine but also inhibits the harmful substances that are transferred from the intestines to the blood. Additionally, *Bifidobacterium* species promote ammonia elimination during the blood transfer to the intestinal tract, which reduces the possible damage to the liver, kidney, heart and other organs that may be affected, thus delaying the aging process.

Lactobacillus colonisation in the intestine is normally harmless, synthesising vitamins, aiding in food digestion and nutrient absorption, promoting the primary metabolism of the host, overcoming the process of corruption, lowering blood cholesterol and increasing endurance by increasing the production of lactic acid in the host. Due to the differences in species, *Lactobacillus* may have different physiological effects. The physiological role of *Lactobacillus* is only next in importance to *Bifidobacterium.*

Bacteroides is one dominant flora in the intestines of many animals and account for almost 32 % of all viable organisms that have been isolated from the gut. *Bacteroides* is Gram-negative and nonspore bacillus, which either cannot movement or move with peritricha. Additionally, *Bacteroides* is an obligate anaerobe and organic heterotroph, which uses carbohydrate, peptones or intermediate metabolites. Some *Bacteroides* species have a positive influence on host nutrition, immunity, metabolism, growth and development, as well as many other aspects of aging. However, some species are opportunistic pathogens.

Peptococcus and *Streptococcus* are Gram-positive obligate anaerobes and have some of the largest numbers of flora that are housed in the animal gut. One gram of stool can have up to 10^{10}–10^{11} cfu g^{-1} bacteria. The physiological role of these anaerobes is poorly understood (Chaveerach et al. 2004).

Ruminants are a rather special group of mammals, whose diet is primarily composed of plant materials. Rumination not only reduces the particle size of the fibres to expose sugars for microbial fermentation but also neutralises acids that are produced during microorganism fermentation by buffer substances (carbonates and phosphates) in the saliva, which maintains an neutral acidic environment that is conducive to fibre degradation and rumen microbial growth.

Different from monogastric animals, the stomach of ruminants consists of four parts, which are the reticulum, rumen, omasum and abomasum. The rumen, which is the feed processing plant of the ruminant body, is a unique digestive organ of ruminants, and approximately 70–85 % of digestible matter and 50 % of the crude fibre of the feed is digested in the rumen. Thus, rumen (including the reticulum) digestion occupies an important position in the digestion process of ruminants. The rumen and reticulum are the two stomachs that have high-density microbiomes (bacteria, protozoa and fungi). For nonruminant animals, the intake food is digested in the stomach first, but the chyme in the stomach of ruminants mainly consists of feed particles that are not fermented, some fermentation products and microbial organisms that grow in the rumen.

The rumen has highly anaerobic conditions, and the anoxic environment is conducive to the growth of some specific types of bacteria, including the strain that may degrade plant cell walls (fibre) for the simple sugars (glucose) and microbial fermentation of glucose to obtain energy for maintaining their growth.

Microbial flora in the rumen are complex and often change due to foraging species, feeding times, individual differences and other factors. Rumen microorganisms primarily include bacteria, protozoa (including flagellates and ciliates) and fungi; however, anaerobic ciliates and bacteria are primarily involved in digestion and their type and number are the greatest. Approximately 15–25 billion bacteria and 60–1,000,000 ciliates are contained in 1 g of rumen contents, which occupy approximately 3.6 % of the total volume of rumen fluid and bacteria and ciliates occupy 50 % (by volume). However, concerning the metabolic activity intensity and the importance of its role, bacteria are far greater than ciliates.

There is a variety of rumen ciliates and there are no uniform classification criteria thus far. Generally, rumen ciliates are divided into two categories, *Holotricha* and *Entodiniomorphida*. Anaerobic ciliates primarily include *Isotricha* and *Dasytricha* of *Holotrich* and *Diplodinium*, *Entodinium* and *Ophryoscolex* of *Ophryoscolecidae*. Most ciliates are within *Holotrich* and *Diplodinium*, which account for 58–98 % of the total number of ciliates and *Diplodinium* are primarily capable of breaking down cellulose.

The many different types of rumen bacteria that can break down cellulose include *Ba. succinogenes*, *Clostridium cellobioparus*, *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Ruminobacter parvum*, etc. Additionally, those rumen bacteria that can ferment starch and sugar include *S. bovis*, *C. butyricum*,

Butyrivibrio sp., *Selenomonas ruminantium*, etc. Moreover, rumen bacteria that can synthesise proteins include *Amylococci*, *Amylosarcinae*, *Amylospirillae* and other microorganisms that are addicted to iodine. Furthermore, rumen bacteria that can synthesise vitamins are *Flavobacterium vitarumen*, *Clostridium* and so on.

From a phylogenetic perspective, *Methanogens* belongs to the *Archaea* genus; this genus is different from "true bacteria", which are strictly anaerobic, the composition of the cell wall does not contain peptidoglycans and the cell membrane contains false murein, proteins, glycoproteins or heteropolysaccharides, with special coenzymes, such as CoM and F_{420}. By comparing the size of the 16S rRNA nucleotide sequence, the similarity (homology), which is the SAB value that is present in cells of methane bacteria, these bacteria can be divided into the following five categories: *Methanobacteriales*, *Methanococcales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanopyrales*.

Flagellates always exist in newborn ruminants; however, the number reduces with increasing age, different types of feed intake (FI), and changes in the feed composition.

Since 1975, when Orpin first proved that fungi exist in the rumen, people have performed more in-depth research (Orpin and Joblin 1997). By 2000, 15 types of rumen anaerobic fungi had been isolated. According to the form of free spores and the manner of mycelia formation, these anaerobic fungi are divided into two types, specifically, multi-centre and single-centre fungi. Multi-centre fungi primarily include two genera, *Orpinomyces* and *Anaeromyces*, and single-centre fungi include three genera, *Neocallimastix*, *Promyces*, and *Caeomyces*.

Rumen microorganisms vary, and the role and function of these organisms is extremely complicated. Many rumen microorganisms remain that have not yet been isolated and identified. Additionally, the roles, functions, and other aspects of these organisms must be further studied.

7.1.3 Lactic Acid Bacteria and Animal Immunity

In recent years, there has been a growing interest in the animal immune system because of the economic importance of animals as livestock and because some animals may be used as models for the human immune system. The activation of systemic and secretory immune responses by LAB requires many complex interactions among the different constituents of the intestinal ecosystem (microflora, epithelial cells, and immune cells). LAB are a major part of the commensal microbial flora of human and animal GITs (Barrington et al. 2002). In addition, LAB not only influence the large intestine by affecting the intestinal flora but also by affecting other organs via the modulation of immunological parameters and intestinal permeability or via the production of bioactive or regulatory metabolites. Dietary supplementation with special LAB would promote health and reduce the risk of various diseases by enhancing the bioavailability of nutrients and by being beneficial to the regulation of intestinal microflora and immunomodulatory

properties. Moreover, with the ability to avail immunity, LAB would be used to reduce microbial infection and to induce immune responses, enhance the immunity, and respiratory immunity of the host, alleviate pollen allergic reactions and improve food digestion in immunocompromised hosts.

7.1.3.1 Reduce Microbial Infection

At present, the common method to treat infections that are caused by pathogenic microorganisms is the injection of antibiotics. However, this method could cause problems, such as coliform disorders, double infection, and antibiotic residues. Therefore, a new method to prevent the abuse of antibiotics is required, and it seems that using probiotics with antagonistic activities to reduce microbial infection would be a perfect solution. LAB administration could lead to significant changes in the intestinal microflora and contribute to the maintenance of colonisation resistance, primarily against *Listeria* sp., *Escherichia* sp., and *Salmonella* sp. (Havenitha et al. 2002; Castillo et al. 2012), which can disturb the normal intestinal microflora and lead to gastrointestinal disorders, resulting in diarrhoea. Some studies have showed that these two pathogens are the most frequent bacterial etiologic agents in calf scours during the first week of life (Barrington et al. 2002; Millemann 2009; Signorini et al. 2012). The ability of LAB to affect immune responses suggests that these microorganisms would contribute to the recovery from infections. Moreover, there is evidence that some LAB enhance immunomodulatory mechanisms through inducing a high level of specific secretory IgA, which is a major immunological barrier against viruses. Thus, LAB have been used for the development of probiotic foods and animal feed additives.

7.1.3.2 Induce Immune Responses

Some types of LAB are able to induce specific secretory immunity, and others can enhance the gut inflammatory immune response. The immunomodulating capacity of LAB, together with the possibility of targeting antigens to specific sites of the bacterium, offers attractive opportunities for the treatment of infectious diseases through vaccination and of autoimmune diseases or other immune disorders by modulating the immune response in a directed and predetermined way. Generally, the anti-LAB antibody is related to the processing and presentation of microorganisms to immune cells. Some strains of LAB have been proven effective in inducing protective immunity and in improving the immune response. Maldonado et al. (2007) demonstrated that LAB showed an increase in IgA, IL-10, and $CD4^+$ cells. Additionally, the pro-Th1 immunomodulatory strains *L. casei* Shirota and *L. plantarum* have been shown to downregulate the allergenic molecules IL-4, IL-5, and IgE in antigen-primed mice by oral delivery in viable form Matsuzaki and Chin (2000).

However, the potentially important immunoregulatory role of LAB in allergy treatments and in prophylaxis should not be discounted, particularly because feeding supplementation may represent a safe, noninvasive means of immunotherapy.

7.1.3.3 Improvement of Respiratory Immunity

LAB have been utilised for the development of probiotic feeds with the ability to stimulate respiratory immunity, which would increase resistance to infections, even in immunocompromised hosts. Some studies have demonstrated that the immunomodulatory activity of LAB might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well. Thus, LAB represent a promising resource for the development of prevention strategies against respiratory infections, which could be effective tools for medical applications. LAB are able to induce the activation of the systemic innate immune response, which is evidenced by increasing the number and microbicidal function of blood neutrophils. Some reports have indicated that IL-10 would be valuable to attenuate inflammatory damage and pathophysiological alterations in lungs that are infected with pneumococci. According to these results, LAB treatments would beneficially regulate the balance between TNF-α and IL-10, which would allow a more effective inflammatory response against infection.

7.1.3.4 Anti-allergy Properties

LAB decrease the prevalence of allergies in susceptible individuals, inhibit the inflammatory responses in the gut and have antagonistic effects against intestinal pathogens. Although most research concerning LAB-mediated enhanced immune protection is focused on GIT pathogens, LAB have been considered to offer clinical benefits to allergy sufferers. However, since the last decade, there has been accumulating evidence that LAB can alleviate some of the symptoms of atopy, such as their ability to liberate de novo immunoregulatory peptides from major food proteins via enzymatic hydrolysis; this mechanism has been demonstrated experimentally for some strains of LAB, and in one case, this mechanism has been shown to be effective at reducing ex vivo IL-4 production by peripheral blood lymphocytes of atopic patients (Matar et al. 1996; Rokka et al. 1997; Sutas et al. 1996). Moreover, LAB are able to enhance immune system development, limit allergy development, and prevent the overexpression of an allergic phenotype in the early life of animals.

7.1.3.5 Stimulate the Innate Immune System

Generally, LAB induce the up or down regulation of the innate response to maintain intestinal homeostasis. LAB strains with immunomodulatory properties are able to stimulate the innate immune system. Therefore, LAB would be based primarily on the induction of an early intense innate immune response together with an improved ability to regulate the inflammatory response that prevents tissue damage. Julio et al. (2011) showed that some LAB were able to accelerate the normalisation of the immune response to infection in malnourished mice. According to their results, the use of probiotic bacteria was associated with a pattern of inflammatory and anti-inflammatory cytokines that led to a more efficient regulation of the inflammatory response.

This section discusses the influence of LAB on the immune system of animals. Depending on the interaction between LAB and the intestine, the immune response that is obtained will be different at different mucosal sites. Despite the requirement for multiple screenings to verify the immunopotentiator activity of LAB, their behavior in complex interactions within the intestinal ecosystem and their influence on the immune cells can be predicted. Thus, if some LAB induce IgA^+ B cell and $CD4^+$ T cell migration, then it is believed that this LAB would have local and systemic effects. In contrast, it is considered that LAB only play a role in the gut and could be used to increase intestinal mucosal immunity. In general, the intestinal immune systems of hosts balance defence with immunological tolerance, including responding to pathogens, while coexisting with resident bacteria and food antigens. The host body has an "immune system" to recognise pathogenic bacteria and viruses and then to act to remove these pathogens. LAB and their components can act on the immune system within the body and, in many cases, enhance the immune response.

7.1.4 Lactic Acid Bacteria and Animal Health

In recent years, there has been a considerable interest in using some probiotic microorganisms (LAB) to replace the use of antibiotics in feeds. The ability of probiotic microorganisms to inhibit or, at least, to counteract the negative effects of pathogens in live animals has been well studied in many animals. LAB are common components in normal intestinal microbiota, in both human beings and domestic animals. In this chapter, we will use young calves, weaned piglets, chickens, canines, and fish as examples to discuss the close correlations between LAB and animal health.

7.1.4.1 Calves

Currently, there is an increasing trend of beef demand all over the world. However, calf mortality due to diseases causes serious losses to beef industry. Before weaning, dairy calves are susceptible to many pathogens, which can affect their subsequent performance. The use of LAB has been identified as a tool to maintain the intestinal microbial balance and to prevent the establishment of opportunistic pathogenic bacteria populations. Neonatal calf diarrhoea primarily affects animals under 6 weeks of age, and *Salmonella* spp. and *E. coli* are the most frequent bacterial etiologic agents in calf scours during the first week of life. LAB have been used to control the effects of pathogens, such as *Salmonella* spp. and *E. coli*. Bovine mastitis is a costly infectious disease of dairy cattle, which is responsible for significant economic losses all over the world. Additionally, mastitis has affected antibiotic resistance. Lee (2007) screened 42 LAB strains that had a broad spectrum of antimicrobial activity against mastitis pathogens. Symbiotic bud bacteria, which are a type of LAB that were originally artificially cultivated on intestinal tracts of calves, can successfully inhibit the growth of livestock and poultry intestinal pathogenic organisms, as reported by Australian researchers (Lin 1993). The use of lactose was able to generate an increase in the number of the *Lactobacillus* genus. The explanation for the favourable influence of lactose on the presence of intestinal LAB is that when lactose is present, even in small quantities, the best environmental conditions are created for these particular organisms, without a positive change in the other bacteria that are normally present. The calves that are inoculated with probiotics may have a better advantage of lactose and, thus, have a better growth performance. These results show that LAB can be used as probiotics for calves.

7.1.4.2 Weaned Piglets

Weaning is one of the most critical events in pig production because this process involves major changes in feeding, management, and the environment. Moreover, these piglets also suffer the trauma of separation from the sow, as well as a change in housing, which can also contribute to an increased stress status. The change in diet may alter the gastrointestinal microbial balance, which increases the opportunities for pathogens to colonise the gut. In addition, weaning often results in dramatic changes in the intestinal morphology and gut function, such as villous atrophy and crypt hyperplasia, which are thought to cause a decrease in the digestive and absorptive capacity of the small intestine.

To improve pig performance, as well as to help pigs resist common diseases that occur in hot and humid climatic conditions, the feed regulations allow the use of some antibiotics as feed additives. However, this addition of antibiotics is a controversial issue because the misuse of antimicrobials could promote bacterial resistance and result in less efficient antibiotic treatments for human and animal diseases. Thus, there is an urgent requirement to remove antibiotics from the list of

feed additives that are allowed for food-producing animals. The provision of probiotic bacteria has been suggested to improve the performance and health of weaned piglets and is one of the alternatives to the use of antibiotic feed additives. Giang et al. (2010) found that, in the first two weeks after weaning, piglets that were fed diets that were supplemented with LAB complexes were less affected by diarrhoea than piglets that were fed the control diet, which suggested that the LAB complexes that were tested have probiotic properties in weaned piglets. Therefore, LAB have a potential value as feed additives for pig feeds.

7.1.4.3 Chicken

The use of probiotics as feed supplements for the control of pathogen infection and for the enhancement of immune responses in chickens has been reported. Chickens may harbour pathogens, such as *Salmonella typhimurium* and *Sa. enteritidisin*, in their intestines, and the oral administration of live LAB cultures in the form of probiotics may reduce the intestinal colonisation of *Salmonella* in chickens. In addition, probiotics may offer other functions, such as the maintenance of health and the promotion of the growth of chickens. Chen et al. (2012) reported that multistrain probiotics that consisted of LAB strains that were selected by their immunomodulatory activity and adherence are more effective than those probiotics that consisted of strains that were selected at random in antagonising *Salmonella* colonisation, invasion, and induced inflammation.

The selection of probiotic efficacy included aggregation, antibacterial effects, enzymatic activities, cell surface hydrophobicity, coaggregation and tolerance to bile salts, and acidic conditions. The tests of aggregation and cell surface hydrophobicity could be used to examine the adhesion ability of LAB to mucus. LAB can reduce the number of pathogens in the GIT by producing bacteriocins, H_2O_2 and, particularly, organic acids. Therefore, the antibacterial effects of LAB were investigated as a second step. Coaggregation is thought to be linked to the ability to interact closely with undesirable bacteria. This attribute is another characteristic that represents the competitive exclusion of LAB against enteric pathogens. The resistance to acidic pH and bile salts is of great importance in the survival and growth of bacteria in the intestinal tract and, thus, is a prerequisite for choosing suitable probiotics. These bacterial attributes, when evaluated together, could lead to the selection of an organism that might be different from those bacteria that have been isolated in other studies, and the efficacy of a selected probiotic in improving health and digestion might be enhanced.

7.1.4.4 Dogs

Puppies were fed dog food that was mixed with LAB that were isolated from intestines and faeces of canines, and no negative effects on health occurred, which demonstrated that the dog-derived *lactobacillus* had beneficial effects on dogs and

could be used as a probiotic preparation for dogs. After being fed *lactobacillus*, there was a significant reduction in the number of *E. coli*, an increased number of beneficial bacteria. Additionally, the digestion rate of food was also greatly improved. Dogs with gastrointestinal diseases were orally fed *lactobacillus* ADI, which can reduce high levels of cholesterol and alanine aminotransferase in the blood. *Lactobacillus acidophilus* DSM13241 can improve the intestinal flora composition of healthy dogs, strengthen their digestive health and improve their immune function.

Rinkinen et al. (2000) studied the adhesion of LAB to canine small intestinal mucus and their adhesive ability after pretreatment with jejunal chime, and the results indicated that probiotic strains of human origin, which were intended for human use, adhered to canine intestinal mucus, warranting the further investigation of these strains for use in dogs. Intestinal mucosal adhesion is one of the main selection criteria for probiotic microorganisms. This property is considered important for transient colonisation, antagonism against pathogens, modulation of the immune system and enhanced healing of damaged gastric mucosa.

7.1.4.5 Fish

Most probiotics primarily play roles in preventing gastrointestinal diseases, improving digestion and absorption and stimulating the immune system. However, for aquatic animals, the functions of probiotics also include surviving in water and increasing the types of beneficial bacteria, such as *Lactobacillus*, *Bifidobacterium*, *Vibrios*, *Pseudomonas*, *Bacillus*, nitrifying bacteria and photosynthetic bacteria (Yang 1999).

Commercial probiotics seem to improve the dietary value of rotifers for flatfish larvae; however, the effect of individual strains of bacteria remains unclear. Other roles remain to be investigated, such as a possible competition for nutrients with pathogens in the digestive tract or the hypothetical stimulation of the immune system. The stimulation of the immune system of terrestrial animals by LAB has already been demonstrated. Nonspecific defences are stimulated by *L. casei* in mice, e.g., the activation of macrophages, as assessed by lysosomal enzyme activities. The production of the immunoglobulin IgA is also stimulated, which protects mice against *Sa. typhimurium*. Fermented milk that contains LAB may serve as an adjuvant in vaccinations against *V. cholerae*. The serum and intestinal immune responses against rotavirus are promoted in children that ingest *L. casei*. Anticarcinogenic effects have also been shown. These aspects deserve further attention in fish, particularly for developing vaccines against bacteria and viruses or for limiting the effect of toxic compounds that cause carcinomas. For instance, the oral ingestion of a peptidoglycan that is derived from *B. thermophilum* improved the resistance of yellowtail against *En. seriolicida*.

Although LAB are not dominant in the normal intestinal microbiota of fish due to variance with homeotherms, some strains can colonise the gut. However, it is possible to artificially maintain LAB populations at high levels by regular intake

with food. The applications of such treatments should be further considered to improve the health and quality of fish in culture. In contrast, there are a growing number of studies that have implicated a role for LAB in fish diseases, although vaccination is not yet fully operational. One of the most promising and urgent applications of harmless LAB is, therefore, to investigate their use as both probiotics against gram-positive pathogens and sources of immunostimulants.

7.1.4.6 Conclusions

This review discusses the use of LAB in animal feeding. The protective effects of LAB that can inhibit food-borne pathogens in live animals before slaughter have been discovered. LAB are capable of producing inhibitory substances, other than organic acids, that have inhibitory activities against different microorganisms. However, not all LAB will reduce food-borne pathogens in farm animals; therefore, the careful selection of strains to be administered under appropriate conditions is important.

7.1.5 Lactic Acid Bacteria and Animal Production Performance

Many microorganisms colonise the GIT and establish a stable, dynamic equilibrium relation with the host, which has an important role in animal health. Since the twentieth century, the use of antibiotics and other feed additives have disrupted the micro-ecological balance in animals, which has led to digestive disorders and caused various diseases. To address the drawbacks that have been caused by the use of antibiotics, there is a requirement for additives with nontoxic side effects, no residue, no drug resistance, low cost, and significant effects, and probiotic animal feed supplementation has risen as a viable alternative to antibiotics, as reported for several monogastric or ruminant farm animals.

The more widely accepted concept of probiotics is “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host” (Joint FAO/WHO Expert Committee 2002). Probiotics are primarily composed of LAB and bifidobacteria, which form part of the normal enteric flora of humans and animals, and probiotic feed supplementation may directly benefit the animal host by preventing infection and by combating the causative agent of intestinal disorders or indirectly benefit by balancing the disrupted equilibrium of the enteric flora and by augmenting the host’s immune responses.

Studies have shown that LAB affects the antagonistic activity of the intestinal pathogen. Aerobic bacteria that colonise in the intestines of humans and animals are potential pathogens, and anaerobic bacteria play an important role in controlling potential pathogens. LAB are important anaerobic bacteria in animal

intestines, which are the normal intestinal colonisation bacteria of the animal, that reduce the intestinal pH through producing lactic acid and other acidic substances from metabolism; lowering the redox potential; stabilising normal flora by generating hydrogen peroxide and other antimicrobial substances, such as bacteriocin; competing for nutrients and attachment sites with other pathogenic bacteria to reduce the ability of pathogen infection. Therefore, LAB become excellent bacteria to prepare as inoculants. Multiple studies have indicated that including lactobacilli in animal feed can affect the ecological balance of intestinal flora. In addition, LAB have become widespread in milk and dairy products. Several probiotic strains have been utilised in fermented sausages, including lactic acid-producing bacteria that primarily belong to the genera *Lactobacillus*, *Pediococcus* and *Streptococcus*. The *Enterococcus* genus is of particular medical relevance because of its increased incidence as a cause of disease in hospital-acquired (nosocomial) infections and in acquired antibiotic resistance toward available antibiotic therapies. Several virulence factors have been described, and the number of vancomycin-resistant enterococci is increasing (Leavis et al. 2006). Strains that belong to *En. faecium* have a long history of apparently safe use in industrial and agricultural applications; however, other species, such as *En. durans* and *En. hirae*, have been associated with infections in chickens (Abe et al. 2006). The use of enterococci as probiotics remains a controversial issue. Although the probiotic benefits of some strains are well established, the emergence, and the increased association of enterococci with human diseases and multiple antibiotic resistances have raised concerns regarding their use as probiotics.

The GITs of newborn animals are sterile, and with contact with the surrounding environment, through the mother, air, water, breast-feeding, grazing, and other ways, huge microbial evolution communities gradually form through the GIT. Studies have suggested that the artificial inoculation of LAB in newborn animals, when their GIT has not yet formed microflora, allows beneficial LAB to occupy the intestinal environment first, thereby reducing the chances of infection by antagonistic pathogens. LAB diets for weaned animals have a positive effect on promoting intestinal microflora balance, reducing the incidence of diarrhea and improving immunity. Diets that are supplemented with LAB can not only enhance the production performance of fully developed animals but also play an important role in improving the quality of livestock housing and in reducing malodorous gases.

The supplementation of ruminant feed with LAB has a beneficial effect on subsequent milk yields, fat, and protein contents. Blood and milk parameters were significantly improved using LAB, as shown by higher serum cholesterol and total lipid concentrations and by higher milk fat and protein contents at the mid-suckling period in sows. In traditional milk products, microbes are selected for their ability to grow and produce organic acids in milk. In the case of probiotic products, the microbes are primarily selected because of their potential health-associated properties. The number of viable microbial cells that should be present in a probiotic product has been considered to be between 10^6 and 10^8 cfu/ml.

LAB increased egg production and egg quality and decreased egg contamination. LAB also increased eggshell weight, shell thickness and serum calcium. In addition, LAB significantly reduced the plasma cholesterol and triglyceride levels, which confirmed the important roles of GIT microorganisms in lipid recycling. Probiotics had no effect on chick quality or on production efficiency in broilers that were produced by the breeder flock.

As probiotic agents, bifidobacteria have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders, such as colonic transit disorders, intestinal infections, colonic adenomas, and cancer. Bifidobacteria are promising probiotics, although probiotic properties are species- and/or strain-specific. Bifidobacteria are able to prevent or alleviate infectious diarrhoea through their effects on the immune system and their resistance to pathogen colonisation. There is some experimental evidence that certain bifidobacteria may actually protect the host from the carcinogenic activity of intestinal flora. Bifidobacteria may exert protective intestinal actions through various mechanisms and represent promising advances in prophylaxis and therapy. Bifidobacteria are frequently used in food and pharmaceutical preparations, and their application in animal feeding is increasing. *Bifidobacterium* species are widely applied as probiotics in humans; however, only few studies (and often together with *Lactobacillus*) have been conducted in animals, particularly in pigs. *Bifidobacterium animalis* demonstrated high survival in the small and large intestines when ingested in a fermented dairy product. *Bifidobacterium infantis* has been shown to exert a broad spectrum of antimicrobial properties through the production of antimicrobial compounds, which are unrelated to acid production, that inhibit the growth of pathogens.

In animal feed additives, few probiotics with only *Bifidobacterium* have been reported; however, some mixed application formulations use *Bifidobacterium*. Lactobacilli and bifidobacteria administration immediately after birth promote the colonisation of beneficial commensal microbiota that are capable of limiting artificial formula-induced mucosal atrophy, dysfunction, and pathogen load in premature neonatal piglets, thereby reducing the incidence and severity of necrotising enterocolitis and lowering the colonisation density of the potential pathogen *C. perfringens* (Siggers et al. 2008). Probiotic preparations that included *B. lactis* and *L. rhamnosus* individually reduced the adherence of *Salmonella*, *E. coli* and *Clostridum* spp. to the intestinal mucosa in swine. Reduced mucosal adhesion by pathogens is presumed to lead to the reduced severity of clinical diseases. *Bifidobacterium animalis* ssp. *lactis* positively affected the growth performance of weaning piglets and the ratio of bifidobacteria to *E. coli* in the gut (Modesto et al. 2009). Cheese was optimised by the addition of *B. lactis* and *L. acidophilus*. Quarg, which is produced by LAB, has shown a beneficial effect on intestinal function and on the promotion of good health.

The expected health-promoting criteria are the regulations of intestinal microbial homeostasis, the stabilisation of the gastrointestinal barrier function, the expression of bacteriocins (Mazmanian et al. 2008), enzymatic activities that

induce absorption and nutrition (Timmerman et al. 2005), immunomodulatory effects and the inhibition of pathogens that colonise and infect the mucosa.

7.2 Effects of Lactic Acid Bacteria on Feed Preparation and Animal Production

7.2.1 Poultry

With increasing concerns regarding antibiotic resistance, the ban on subtherapeutic antibiotic usage in Europe (Anadón et al. 2006) and the potential for a worldwide ban, there is increasing interest in finding alternatives to antibiotics for poultry production. The most common types of probiotics that are available are LAB. Disturbances in the intestinal microbiota can increase the susceptibility to infection. Numerous in vivo and in vitro studies have shown that the addition of LAB inhibit pathogens and increase infection resistance. Therefore, it would seem effective to use LAB cultures following antibiotic withdrawal from the diet to promote the reestablishment of a favourable microbial population in the digestive tract.

7.2.1.1 Tests for LAB Screening

LAB can colonise the alimentary canal surface only if these bacteria can multiply in the prevailing environmental and nutritional conditions. How to screen LAB that are useful for poultry production is worth consideration. Tests that are used for screening should be simple, rapid, and comprehensive. The study by Taheri et al. (2009) provided a good method for us to reference. The aggregation test is proposed to be appropriate for the first step of screening because this method is simple and applicable to many test strains. Because colonisation is an important characteristic, the ability to adhere to mucosa is also examined by testing cell surface hydrophobicity, in addition to aggregation. At the second step of screening, antibacterial activities should be considered. This characteristic indicates another aspect of the competitive exclusion of LAB against enteric pathogens. The aggregation time and antibacterial activity demonstrate the ability of LAB to prevent the colonisation of *E. coli, Salmonella* and other enteric pathogens. Furthermore, the enzymatic activities of LAB are considered in the screening. A probiotic that has enzymatic activities (the expression of α-amylase, phytase, β-glucanase, xylanase, or cellulose enzymes) can improve digestion, particularly in newly hatched chicks. The ability of LAB to closely interact with pathogens, such as *E. coli*, would constitute an important host defence mechanism against infection, therefore, the coaggregation test is also performed. However, the results of Taheri et al. indicated there was no coaggregation between LAB and enteric

strains. The effects of bile salts and acidic conditions on the survival of LAB should be investigated. Concerning the pH and retention time of feed in the GIT of the chicken [crop (pH 4.5), 30 min; proventriculus (pH 4.4), 15 min; gizzard (pH 2.6), 90 min; small intestine (pH 6.2), 90 min; and large intestine (pH 6.3), 15 min, respectively (Church and Pond 1974)], the examination of the survivability of the strains that are close to the retention time of feed and to the pH in the gizzard is preferred. As a source of potential probiotic, LAB should be chosen for the following attributes: high cell surface hydrophobicity, coaggregation, high-enzymatic activities, particularly phytase and amylase activities, and resistance to bile salts and acidic conditions.

7.2.1.2 Feed Preparation

The reproducible delivery of LAB in poultry production is problematic because the maintenance of viability is key to their beneficial activity but is difficult to achieve with commonly used feed processing technologies. During the usual method, LAB strains are cultured with MRS, and inoculated in a fermentation medium, such as diluted cheese whey (Fajardo et al. 2012), skim milk powder or even vegetable or grass juice. Subsequently, the cultures are mixed with poultry feed or are directly delivered in drinking water.

7.2.1.3 Effect of LAB on the Health and Growth of Poultry

Fajardo et al. (2012) studied the effects of the supplementation of both preparations (containing live LAB: *Lactococcus lactis* CECT 539 or *L. casei* CECT 4043) and an antibiotic (avilamycin) on body weight gain (BWG), FI, feed consumption efficiency (FCE), relative intestinal weight and intestinal microbiota counts of broiler chickens. These probiotic preparations were added weekly to the basal diets of broiler chickens. One-day-old males were used in both experiments and were fed ad libitum. In experiment 1, 120 medium-growth Sasso X44 chickens were used for an experimental period of 42 days. At the end of the experiment, no differences were found for FCE (g of FI g^{-1} of BWG) among groups, and the consumption of different diets did not significantly affect the final coliform counts in Sasso X44 chickens. However, counts of LAB and mesophilic microorganisms were higher in the animals that received the two-probiotic preparations. In experiment 2, 1,200 commercial Ross 308 broiler chicks were used for an experimental period of 31 days. At the end of the experiment, no differences in BWG were observed among groups. However, broilers that were fed avilamycin had the highest FCE. At 16 days of treatment, broilers that were fed the probiotic preparations had the lowest FCE. Although the FCE values increased in all treatments at the end of the experimental period, broilers that were fed the *L. casei* CECT 4043 preparation were the most efficient at converting feed into live weight.

The results showed that adding LAB as a probiotic to the diet could improve the feed efficiency.

In contrast to beneficial responses of probiotic supplementation to broiler diets, Buenrostro and Kratzer (1983) found that broilers that were grown in battery brooders and that were fed a diet that contained a LAB culture did not perform as well as control birds or as those birds that were fed antibiotics. Various levels of biotin were fed to broiler chicks to determine the effect on the biotin status of chicks. The LAB groups were fed a diet that was similar to the control groups; however, chicks were inoculated on alternate days with a commercial *L. acidophilus* culture (1×10^8). This inoculation resulted in a significant decrease in growth and liver biotin in chicks that were fed a diet that was marginally deficient in biotin. Watkins and Kratzer (1983a, b) reported that lactobacilli did not decrease liver biotin in broilers that were fed a practical diet that was adequate in biotin. These results implied that the poor performance of inoculated chicks was a result of competition for biotin between the host and microorganisms. From these data, it would seem advisable to ensure an adequate biotin status in the diet if LAB cultures are fed to chickens. A proper level of lactobacilli may be required by the chicken that provides the most host benefits. Dosing below or above this optimum level may result in undesirable effects, such as bacterial competition for biotin.

7.2.1.4 LAB Use in Prophylactic and Therapeutic Treatments

Salmonella enterica serovar Heidelberg (SH) has been recognised as one of the most common serovars that are associated with foodborne infections in several countries around the world. SH has shown resistance to various antimicrobial agents. Sources of human SH infection include the consumption of undercooked poultry or eggs and egg-containing products. A series of experiments was conducted by Menconi et al. (2011) to evaluate the ability of a commercial probiotic culture (FloraMax, IVS-Wynco LLC, Springdale, and AR), which was composed of eleven LAB isolates of poultry gastrointestinal origin, to reduce SH in chicks and turkey poults. The commercial product (FloraMax) was diluted in reconstituted powdered skim milk to a final concentration of 4×10^6 cfu/ml. Day-of-hatch male broiler chicks or turkeys were challenged via oral gavage (0.25 ml), with approximately 10^5 or 10^6 cfu/chick and 10^6 cfu/poult of SH, and then treated 1 h following SH challenge with approximately 10^6 cfu/chick or 10^6 cfu/poult of FloraMax culture via oral gavage (0.25 ml). The results indicated that the administration of probiotics significantly reduced the incidence of SH recovery from cecal tonsils of broiler chicks and turkey poults compared with untreated controls at 24 and 72 h following treatment. The results also indicated that turkey poults were more susceptible to SH colonisation than chicks.

The consumption of improperly prepared poultry products continues to result in human intestinal diseases. *Campylobacter* spp., particularly *Campylobacter jejuni*, has been implicated in these aetiologies. Poultry serves as an important reservoir for *Campylobacter* in the food supply and creates a potential health risk. This

microorganism may colonise poultry GITs without deleterious effects upon the birds, and asymptomatic carriers freely spread the microorganisms during production and processing, which results in the further contamination of both live birds and processed carcasses. The reduction of colonisation by food-borne pathogens in live poultry during production should be a goal to reduce consumer exposure. One quantitative risk assessment indicates that the incidence of *C. jejuni* infection in humans could be reduced 30-fold if the number of *C. jejuni* in poultry was reduced 100-fold (Rosenquist et al. 2003). Thus, the effectiveness of experimental intervention measures is being explored. The *L. salivarius* strain NRRL B-30514, which can produce the bacteriocin OR-7 that is sensitive to proteolytic enzymes, was selected from 1,200 isolates of different LAB by Stern et al. (2006) for its anti-*C. jejuni* activity. The purified protein was encapsulated in polyvinylpyrrolidone and was added to chicken feed. Day-of-hatch chicks were challenged with *C. jejuni*. At 7 days of age, the chicks were treated with bacteriocin-emended feed for 3 days. At 10 days of age, the chicks were sacrificed, and the challenge strain was enumerated from the chick cecal content. The results indicated that bacteriocin treatment consistently reduced colonisation by at least 1 million-fold compared with levels that were found in the untreated groups.

Increased bacterial resistance to antibiotics in humans has caused an increase in public and governmental interest in eliminating the subtherapeutic use of antibiotics in poultry. An alternative approach to subtherapeutic antibiotics in poultry is the use of LAB as probiotics. The efficacy of LAB can be influenced by environmental and stress statuses. Research has shown that LAB can be enriched in the intestinal tract by feeding specific carbohydrates (Timms 1968). Identifying LAB for potential probiotics, defining conditions under which these bacteria show efficacy and determining mechanisms of action under these conditions are important for their effective use in the future.

7.2.2 Pig

For the last several decades, antibiotics have been used to promote piglet growth at weaning through the prevention of subclinical and clinical diseases. However, there are increasing concerns in relation to the development of antibiotic-resistant bacterial strains and to the potential of these strains and their associated resistance genes to impact human health. Therefore, there is an ongoing interest in minimising or eliminating the inclusion of antibiotics in pig diets worldwide. Therefore, it is likely that there will be increasing demand in the international market for pork that has been produced without in-feed antibiotics.

Lactic acid bacteria are natural, inexpensive, and safe feed additives that do not endanger the environment with residues and that do not cause bacterial resistance. Due to the effects of LAB, these bacteria could be useful in pig production to improve the well-being of animals, decrease health problems and improve the performance of productive livestock. These benefits could allow producers to

increase their productivity, be in accordance with new food regulations and satisfy consumer demands for safe meat. These products will be highly competitive in the most exigent markets.

7.2.2.1 Feed Preparation

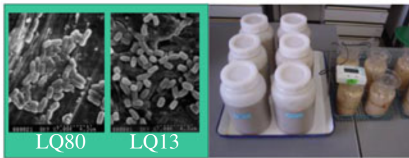
Fermented liquid or solid feed has been used to compensate the use of antibiotics in pig production. Fermentation may occur spontaneously or may be induced. Spontaneously fermented feed appears to be unsafe for pigs. Consequently, the use of specific starters or inoculants to ensure proper fermentation could be a practical solution. In particular, fermenting diets with LAB have been shown to improve the quality of feed and to be beneficial to the health of the animals, and the use of LAB inoculants could be considered a special case of microbial feed additives. Fermented liquid feed is usually made by mixing one part feed (e.g., cereal grains, coproducts, vitamin, and mineral mixtures) with two to three parts water, and fermentation can be achieved in many different ways (e.g., incubation, backslopping, LAB inoculants, etc.). As shown in Figs. 7.1 and 7.2, some fermented liquid feeds were prepared with various food by-products. When the liquid feed inoculated with LAB, the fermentation quality and the microorganism composition in pig intestines were improved (Cai et al. 2007). The moist, solid substrates, in the absence of free flowing water, such as cereal grains, cereal meal or a mixture of grains, and meal, can be used to make fermented solid feed. Solid substrate fermentations are greater productions of biomass when compared with submerged fermentations.

During the preparation of fermented feed, many variables affect the characteristics of the fermented feed that is obtained. Those variables are related to: (1) the specific LAB population that proliferates in the feed, (2) the quantity and type of organic acids that are produced by these LAB, (3) the endogenous population of other microorganisms (competitors) in the feed and their growth potential, (4) the stability of the fermentation temperature (fluctuating temperatures are extremely common in production units) and (5) the addition of organic acids to the feed (instantaneous drop of the pH). There are most likely many other parameters that have not yet been studied or identified. Fermented feed is considered to be of high quality when the fermentation produces stable and high numbers of LAB, a stable and low pH (3.5–4.5) and consequently, a low or nonexistent enterobacteria population.

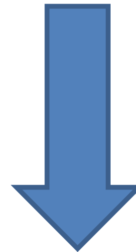
7.2.2.2 LAB Act as Probiotics

Ross et al. (2010) used *L. amylovorus* and *En. faecium*, which were isolated from porcine faeces, as probiotic strains and evaluated the effects of the administration of probiotic strains in pigs (Ross et al. 2010). On the 35th day of age, 3 ml of a mixed probiotic culture (10^8 cfu/ml) was orally delivered by gavage daily. At the end of the fifth week, the pigs that received probiotic bacteria exhibited lower FI

Food by-product collected from local food factory



LAB addition



Fermented liquid and pig feeding

Fig. 7.1 Fermented liquid feed prepared with food by-product and lactic acid bacteria (Cai et al. 2007)

values and better efficiency; however, mean final body weight (BW) values were not significantly different. Adding probiotic culture resulted in significant changes in the enterobacteria population. Compared with samples of the probiotic supplemented feed group, the intestinal tissue of animals in the untreated group exhibited a greater number of eosinophils and widespread cellular infiltration.

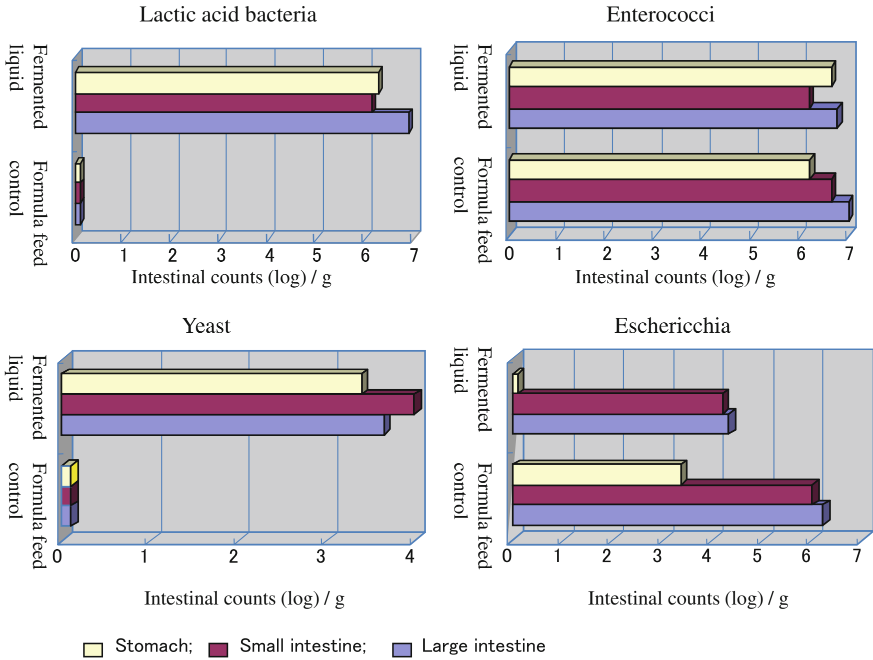


Fig. 7.2 Microorganism composition in GIT of pig (Cai et al. 2007)

These phenomena are usually related to parasite infection, which could cause growth retardation that is characterised by reduced weight gain, or even weight loss, in animals. This study demonstrated the antiparasitic effect of *L. amylovorus* and *En. faecium*.

Suo et al. (2012) investigated the effects of *L. plantarum* ZJ316, which was isolated from infant faecal samples, on pig growth and pork quality. In total, 150 newly weaned pigs were selected for a 60 day treatment (Suo et al. 2012). The results showed that the *L. plantarum* ZJ316 has probiotic effects on pig growth and that the effects of a dose of 1×10^9 cfu/day were more pronounced than those effects of a dose of 5×10^9 cfu/day or 1×10^{10} cfu/day. In LAB-treated pigs (1×10^9 cfu/day), the diarrhoea and mortality rates were lower than in mequindox-treated pigs, the daily weight gain and food conversion ratios were better, and the hardness, stickiness, chewiness, gumminess, and restoring force of pork were all significantly improved. These results showed that *L. plantarum* exerted probiotic effects on pig growth and on pork quality.

The period following weaning is characterised by a high incidence of intestinal disturbances with diarrhea and reduced growth performance in piglets. Post-weaning diarrhoea is usually associated with the proliferation of one or more strains of enterotoxigenic *Escherichia coli* (ETEC) in the GIT. *L. plantarum* was reported to have the ability to decrease diarrhea incidence due to ETEC challenge in weaning piglets (Pieper et al. 2010).

7.2.2.3 LAB Act as Probiotics with Other Types of Strains

Lactobacillus acidophilus, which was isolated from faeces of weaned pigs, as well as *Bacillus subtilis*, *Saccharomyces cerevisiae* (SC) and *Aspergillus oryzae* (AO), were used by Choi et al. (2011) as potential probiotic microbes. These microbes were added to a pasteurised corn:soybean meal (1:1) substrate to perform solid substrate fermentations at different temperatures for 7 days, respectively. The microbial biomass was dried in a forced-air drying oven at low (LT, 40 °C for 72 h) or high (HT, 70 °C for 36 h) temperatures, then mixed and used as potential multimicrobe probiotic products. In total, 288 weaned pigs were fed with different diets for 28 days. The results indicated that the high-drying temperature had no effect on the efficacy of the potential multimicrobe probiotic product. Feeding a 0.60 % probiotic HT diet improved the overall average daily gain (ADG), apparent total tract digestibility (ATTD) of dry weight (DM) and digestible energy (DE) and the *Lactobacillus* population in the faeces and intestines, and it reduced the population of *Clostridium* and coliforms in the faeces (day 14) and in the ileum.

Weaning causes atrophy of intestinal mucosa and decreases IgA protection in piglets, which increases the vulnerability to pathogenic infections. Yoshida et al. (2009) reported the effect of the oral administration of *L. plantarum* Lq80, which is a strain that was isolated from the liquid feed of piglets, in combination with a lactate-utilising butyrate producer, *Megasphaera elsdenii* iNP-001, which was isolated from pig faeces, on the mucosal atrophy that was caused by weaning and on the lowered level of IgA. Twenty-day-old weaned piglets were orally dosed once a day with either (L) 10^{10} cells/dose *L. plantarum* Lq80 or (LM) 10^{10} cells/dose *L. plantarum* Lq80 with 10^{9} cells/dose *M. elsdenii* iNP-001. The two strains were contained separately in capsules. An untreated control (C) was also prepared. After two weeks of administration, *L. plantarum* enhanced the recovery from the villous atrophy. The rectal and colon IgA tended to be higher in L and LM than in C. Colonic butyrate was higher in LM than in the others. The thickness of the colonic mucosa was greater in LM than in the others. These results showed that the effects of *L. plantarum* and *M. elsdenii* were clear in early-weaned piglets.

7.2.2.4 Synergetic Effects of LAB with Other Substances

Salmonella choleraesuis is a host-adapted pathogen of swine. This pathogen is a major serovar for salmonellosis that is transmitted from animals to humans. The study of Chang et al. (2013) suggested that the combined use of *L. acidophilus* LAP5 and *L. reuteri* Pg4 could demonstrate multiple functions, such as preventing pigs from *Sa. choleraesuis* infection, enhancing immunity to prepare host defences against further infection and adjusting the enzymatic activity of intestinal microbes to convert herbal compounds to active compounds. This method offered an alternative way to improve immunity and digestive abilities of animals by using combinations of LAB strains and herbs as feed additives. Trevisi et al. (2008) reported that a combination of *B. animalis* and fructooligosaccharides (FOS)

increased the expression of toll-like receptor 2 in the ileocecal lymph nodes of weaned pigs. Thus, this combination may play a role in enhancing the innate immune response.

LAB that are used as probiotics have been found to improve animal performance, and statistically significant responses have been reported. There have also been reports of no benefit from the use of LAB in pig diets (Walsh et al. 2012; Trevisi et al. 2011). This inconsistency in the results of LAB efficacy might be due to differences in the strain, dosage, sanitary environment, diet type, and in the production stage of the pigs. The establishment of a beneficial LAB population at birth may lead to healthier animals; this establishment may be most effectively achieved by treating sows, which provides an amplification step and floods the neonatal pigs' environment with desirable bacterial strains (Mori et al. 2011). In contrast, it may be necessary to provide a supportive, protective microbiota around the time of weaning because this period is a time of major crisis with instability and a loss of certain bacterial populations (Yoshida et al. 2009).

The reproducible delivery of LAB in industrial pig production is problematic because maintenance of viability is key to their beneficial activity but is difficult to achieve with commonly used feed processing technologies. One specific context where probiotics organisms may be reliably delivered is in systems that utilise fermented liquid feeds. Liquid feed may be fermented by the activity of wild LAB or may be stimulated using specific isolates as "starters"; the latter system has advantages in terms of reproducibility and the speed of fermentation. The farm context in which the organism is used is likely to be critical, and the use of LAB is more likely to result in measurable economic gains in pig production in suboptimal conditions rather than in those pigs that are reared in the highest welfare and husbandry conditions.

7.2.3 Ruminant

7.2.3.1 Silage Inoculant, Fermentation Quality, and Animal Feeding

It is well known that ruminants require continuous and ever increasing forage supply with advancing season for their growth, forage production, however, is seasonal. This often results in a lack of feed availability in seasons when forage is not grown, seriously restricting the productivity of animal husbandry. In order to overcome this problem, producers usually preserve forages at their optimum growth stage for use during those seasons when forage is unavailable. Conserving forage as silage is a better approach than traditional haymaking as there is lesser nutrition loss and lower cost. Silage was used by the ancient Egyptians about 3,000 years ago, and currently has purposes other than merely providing fodder in seasons when forage is unavailable.

Silage can be made by different silos (Fig. 7.3) and is used throughout the year for improving animal performance because it has high nutritive value and good

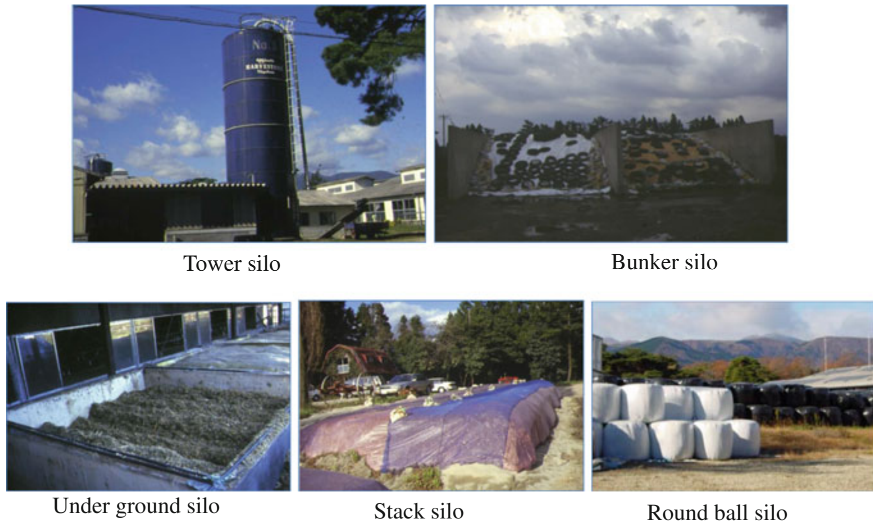


Fig. 7.3 Silo and silage storage (Cai 2001; Cai et al. 2001)

palatability. Ensiling is based on natural fermentation in which epiphytic LAB convert sugars into lactic acid under anaerobic conditions. As a result, the pH decreases and the material is preserved (McDonald et al. 1991). A large variety of forage crops and various moist "by-products" of the food industry can be used as silage materials. The fermentation quality of silage is influenced by the size, diversity and activity of epiphytic LAB, and other conditions (water soluble carbohydrate content, air tightness, temperature etc.). Many LAB have been detected in different silages or at different ensiling stage (Table 7.1). The population density of LAB is reported to range from 10^1 to 10^3 colony forming units (cfu) g^{-1} fresh matter (FM) for standing forage crops, 10^3–10^7 cfu g^{-1} FM for chopped crops entering the silo and 10^5–10^9 cfu g^{-1} FM for the final silage (McDonald et al. 1991; Zhang 2002). However, a large proportion of these bacteria may not be the most effective organisms for promoting a predominantly lactic acid fermentation in the silo or for improving aerobic stability after opening the silo. Therefore, much attention has been focused on the use of LAB as silage inoculants.

LAB Inoculants

Various studies have indicated the advantages of LAB inoculation in silage making, and this process is extensively used globally. Whittenbury (1961) defines the criteria that a potential organism should satisfy for use in silage as follows:

a. It must grow vigorously and be able to compete with and preferably dominate other organisms.
b. It must possess a homofermentative pathway in order to produce the maximum amount of lactic acid from the immediately available hexose sugars.

Table 7.1 Some lactic acid bacteria detected during ensiling

Genus	Species
Lactobacillus	*L. acetotolerans, L. acidophilus, L. brevis, L. buchneri, L. casei, L. cecellobiosus, L. coryniformis, L. curvatus, L. fariminis, L. fermentum, L. graminis, L. lactis, L. nasuensis, L. panis, L. plantarum, L. reuteri, L. rhamnosus, salivarius, L. taiwanensis, L. viridescens, L. zeae*
Pediococcus	*P. acidilactici, P. cerevisiae, P. damnosus, P. dextrinicus, P. lolii, P. parvulus, P. pentosaceus*
Enterococcus	*E. casseliflavus, E. durans, E. faecalis, E. faecium, E. flavesens, E. gilvus, E. mundtii*
Lactococcus	*L. garvieae, L. lactis*
Streptococcus	*S. bovis, S. faecium*
Leuconostoc	*L. cremoris, L. citreum, L. dextranicum, L. lactis, L. mesenteroides, L. pseudomesenteroides*
Weissella	*W. cibaria, W. confuse, W. kimchii, W. paramesenteroides*

c. It must be acid tolerant and capable of producing a final pH of at least 4.0.
d. It must be able to ferment glucose, fructose, sucrose, fructans and, preferably, pentose sugars.
e. It should have no action on organic acids.
f. It should possess a growth temperature range extending to 50 °C.
g. It should be able to grow in material of low moisture content, as might be the case when wilted material is ensiled.

Vanbelle et al. (1985) suggest that it would be beneficial if the LAB used for inoculants were to produce increased amounts of the L(+) lactic acid, given its nutritionally superiority to the D(−) form. In fact, it is difficult for a single strain of LAB to satisfy all the above criteria, of the most inoculants only satisfy some and are selected based on specific requirements. The most important criteria are that a LAB strain must grow vigorously during ensiling and be acid tolerant. A homofermentative strain is necessary when used as a fermentation stimulant, while a heterofermentative strain is more effective as an aerobic deterioration inhibitor. The main LAB used as fermentation stimulants are *L. acidophilus, L. casei*, *L. plantarum, L. rhamnosus*, *Pediococcus acidilactici* and *P. pentosaceus*, which are homofermenters; acid tolerant. *En. faecium* and *S. faecium*, which have weak acid tolerance, are also used in combination with any of above strains. Heterofermentative *L. buchneri* is often used as an aerobic deterioration inhibitor. Recently, there has been a tendency to combine homofermentative strains with *L. buchneri*.

Fermentation Quality

A good silage should have the following characteristics: pH of 4.2 or less, which ensures stability; NH_3–N less than 100 g kg^{-1} total nitrogen; butyric acid less than 10 g kg^{-1} dry matter (DM); and efficient conversion of water soluble carbohydrates (WSC) to lactic acid. Many studies confirm that inoculating

homofermentative LAB improved silage fermentation quality through reducing pH and the ammonia–N and volatile fatty acid content. In contrast, a few studies have shown little advantage in doing this, and some have shown that LAB had a major effect on some, but not all crops. The success of an inoculant as a silage additive depends on many factors, such as the type and properties of the crops to be ensiled, climatic conditions, epiphytic microflora, ensiling technique, and the properties of the inoculant. Zhang et al. (2000) examined the effects of eight LAB strains on silage fermentation quality and found that, when the silages were maintained at 25 °C, all LAB inoculants, either lactobacilli or cocci, improved silage fermentation quality. Of the eight strains, *P. pentosaceus*, *P. acidilactici*, and *L. plantarum* were the most efficient. When the silages were stored at 45 °C, only *P. acidilactici* was effective (Table 7.2). In summary, an inoculant is not always beneficial to all materials or in any conditions; the characteristics of the materials and environmental conditions should be considered when an inoculant is used.

Aerobic Stability

Aerobic deterioration of the silage is undesirable because of associated the high-nutrient losses and possible production of harmful matter. When silage is exposed to air, the microorganisms (yeasts, molds, and bacilli), which retain dormant in the absence of oxygen, multiply and often result in aerobic deterioration. Aerobic deterioration is usually associated with increasing pH, lactic acid decomposition, and heating. Some silages show signs of deterioration in less than 24 h, whereas others remain unchanged and stable during weeks of aerobic exposure. In general, well-preserved silages are considered to be more liable to aerobic deterioration than poorly fermented silages, the latter of which are likely to contain butyric and other volatile fatty acids. These acids, together with ammonia, act as effective preservatives (McDonald et al. 1991). Some studies have shown that inoculating homofermentative *Lactobacillus* spp. restricts aerobic deterioration, but great attention is paid to heterofermentative LAB (mainly *L. buchneri*) in inhibiting aerobic deterioration, and they are extensively used around the world. *L. buchneri* has been confirmed to have beneficial effects for various silages that are prone to aerobic deterioration. A possible explanation is that *L. buchneri* can produce acetic acid from sugars or lactic acid; acetic acid has stronger antifungal properties than lactic acid. In addition, a heterofermentative strain may produce other active material with acetic acid during ensiling.

Animal Feeding

LAB inoculation generally improves silage fermentation, reducing fermentation losses and the contents of ammonia and volatile fatty acids. Therefore, LAB inoculations are also expected to improve the utilisation of silage, such as increasing digestibility, intake, and productivity. The majority of trials confirm

Table 7.2 The effects of LAB inoculations on the fermentation quality of silages (Zhang et al. 2000)

		pH	Acids (g kg^{-1} DM)			Ratio[a]	Ammonia–N
			Acetic	Butyric	Lactic		(g kg^{-1}TN)
	Control	4.47	5.1	20.5	441.	8.6	155.2
25 °C	*Enterococcus faecalis*	4.24	12.0	2.5	56.3	4.7	137.9
	E. faecium	4.19	9.4	0	56.5	6.0	120.7
	E. casseliflavus	4.24	12.0	0	53.0	4.4	129.3
	Weissella paramesenterodes	4.21	16.1	0	51.3	3.2	112.1
	Leuconostoc pseudomesenteroides	4.28	14.7	0	48.1	3.3	112.1
	Pediococcus pentosaceus	3.91	3.5	0	75.3	21.5	120.7
	P. acidilactici	3.83	6.0	0	79.0	13.2	112.1
	Lactobacillus plantarum	3.74	7.0	0	92.1	13.2	77.6
	Control	5.42	4.0	0.5	36.2	9.1	94.8
45 °C	*Enterococcus faecalis*	4.33	5.5	0	41.2	7.5	103.4
	E. faecium	4.33	4.5	0	41.1	9.1	86.2
	E. casseliflavus	4.47	7.9	0	37.7	4.8	120.7
	W. paramesenterodes	4.46	4.5	2.5	41.7	9.3	129.3
	Le. pseudomesenteroides	4.43	3.6	5.0	35.4	9.8	94.8
	P. pentosaceus	4.32	7.2	0	39.5	5.5	77.6
	P. acidilactici	3.88	2.5	0	64.5	25.8	94.8
	Lactobacillus plantarum	4.40	4.5	0	37.9	9.4	103.4

[a] Ratio of lactic acid to acetic acid

these improvements, but some trials do not. This is possibly because the production response of ruminants to inoculants may be influenced by many variables, such as forage type, size of the epiphytic population, inoculant composition, amount and method of inoculant application, DM and WSC contents of the forage, and alterations in aerobic stability. McAllister et al. (1998) reported that a mixed species inoculant of *L. plantarum* and *E. faecium* was more advantageous to digestibility and animal performance than that by the *L. plantarum* inoculant alone. Cai (2001) reported that the intestinal microflora of ruminants was improved by providing LAB-inoculated silage feed, conferring potential benefits to animal health and performance because of the probiotic-like effect.

7.2.3.2 Crop By-product Silage Prepared with Lactic Acid Bacteria

Crop by-product is those products that were made from certain crops, including straw in the field and processing by-products as rice hull and corn cob, leftovers from coarse manufacturing process level of crop. Crop by-product is an important renewable resource and attracted widespread attention in recent years. China has a huge amount of crop by-product, in 2007–2009 average annual amount is 735 million t, which including 198,651,300 t rice straw and 34,382,000 t rice husk, 149,478, 300 t corn stover and 25,716,700 t corn cobs, 143,734,300 t wheat straw,

field of straw that its amount. Rice, wheat, and corn straw totaled 492 million t, plus rice husk and corn cob, three major food crops straw totaled 552 million t (Chinese provincial field crop straw resources field and its time distribution).

In recent years, as China's rural economic development, China's annual output of crop straw, 30 % for agricultural fuel, 25 % for feed, 2–3 % for raw materials of sideline production, 6–7 % back to the field directly, in addition, there are 35 % of the remaining not use rationally. Some cities, suburbs, and major grain producing areas, the demand of living on the straw as a traditional fuel for farmers decreased, resulting in difficult to process residual straw. In order to catch farming and convenience, farmers taken manners of burning straw in the field, which not only waste valuable resources, but causing air pollution, soil salinity, fire, etc. a large number of social, economic and ecological problems.

Ensiling of Crop By-product

Crop by-product in the natural state is poor quality roughage, which in a variety of nutrient content is not balanced with high-crude fiber content, low-crude protein (CP) content and vitamin deficiency. Determine the digestibility of the feed portion of the space occupied shorter digestive tract, affecting FI size. Treatment methods include physical, chemical, and microbial fermentation. After physical and chemical treatment of straw, its palatability and nutritional value are greatly improved, but still cannot be utilised by monogastric animals. Only fermentation by microbial metabolism of specific degradation of the enzyme, when cellulose, lignin and hemicellulose is decomposed into low molecular weight macromolecules such as monosaccharides or oligosaccharides, the nutritional value, utilisation, and feed rate of crop by-product could be improved, palatability enhanced, and FI increased. Usually, silage preparation and storage are considered to be the most effective techniques for fresh straw and stover resources.

Pang et al. 2011, Gabriela et al. (2011) screened, isolated, and identified the LAB from six corn cultivar stovers in Henan Province, China. Isolates were identified biochemically, and at the molecular level using 16S ribosomal DNA (rDNA) sequence analysis. The greater frequencies of recovery of *L. plantarum* and *L. pentosus* strains from corn silage and corn stover samples suggested that these species participated actively in the fermentation process.

Rice straw is important cattle feed resources, and its production capacity is also very great. However, due to the difficulty of collecting, coupled with the lack of efficient technology, so do not use enough effectively. Another reason is the rice harvest in the autumn, when the weather is unstable and cannot be prolonged storage. It is a very important point of development the excellent long-term storage technology, for effective use of this resource. Using rice straw silage as an animal feed has proven economically viable, not only as a way of disposing of rice straw residues but also as a real alternative for feeding livestock in regions where rice is the main crop. Silage quality modulation need to maintain anaerobic conditions, control moisture, lactic acid and soluble sugar content should be sufficient; in

Table 7.3 Fermentation quality and chemical composition of silages

Silage	pH	Moisture (%)	Organic acid (% FM)				Ammonia–N (g/kg of FM)
			Lactic	Acetic	Propionic	n-Butytic	
Corn (Cultivar: Zhongyuandan 32)	3.62	84.76	3.92	0.85	0.41	ND	0.40
Rice (Shuidao 305)	4.07	68.49	1.92	1.05	0.61	ND	0.60
Wheat (Yumai 19)	4.50	72.72	1.25	0.37	0.50	ND	1.80

Values are means of three silage samples
FM fresh matter
Corn and rice silage were stored for 9 months, and wheat silage were for one month

addition, the preparation of rice straw silage, an appropriate modulation method and the storage management are very important. However, rice straw, the content of water soluble carbohydrate (WSC), which is the substrate to ferment lactic acid, is very low, so the natural fermentation and storage cannot be achieved. Thus, the modulation of high-quality rice straw silage, conditions grass of material (moisture, soluble sugar content, and natural attachment of LAB) are very important, if necessary, using appropriate additives, skilled management during storage are extremely important. For fresh rice straw, composition of microorganisms is similar as the composition of feed rice, and compared with maize, aerobic microorganisms is higher, but LAB content is less.

The fresh rice straw silage was prepared by using the round bale system as shown in Fig. 7.4 and Cai et al. (1998) reported that the moisture content of the fresh rice straw material is 65 %, and the content of carbohydrate such as sucrose, glucose and fructose is 3 % (DM%), which is much lower than corn [61]. The content of CP is 4 % (DM%), about 2–3 % lower than the feed rice. the silage inoculated with Chikuso-1 was preserved well with lower pH, ammonia-N and butyric acid, but higher lactic acid content, not inferior to the high-quality forage rice silage (Table 7.3). In addition, even after long storage, there is no growth of fungi, the quality remains good.

Wheat straw is mainly composed of cellulose, hemicellulose and lignin; hence its low digestibility prevents the use in feedlots. The disadvantages for wheat silage is its hollow stem as rice straw, which saving a lot of air, and would result in the massive proliferation of yeast and mold during the process of ensiling, therefore, it is difficult to make good quality silage without inoculant. The addition of cellulase to silage can partially degrade fiber to fermentable WSC used by LAB (Eun and Beauchemin 2008). The mixtures of inoculants and cellulase had also been used to improve the fermentation quality.

Ensiling of crop by-product, especially with proper inoculant, is a simple and low-cost option, which can preserve feeds that are seasonally abundant for later feeding during periods of feed shortage.

7.2.3.3 Tea Grounds Silage, Fermented TMR, and Nutritive Value

Tea is one of the most popular beverages in the world. With the increasing attention on health and nutrition issues in the beverage market, consumption of tea drinks such as green tea has been increasing significantly in recent years. Accompanied with the consumption of readymade tea drinks in bottles, packs, and cans, a large amount of tea grounds are released annually by beverage companies. Although only small amounts of tea grounds are converted into raw compost material, most are generally buried. There is increasing demand for efficient use of food by-products due to economic and environmental concerns (Xu et al. 2007).

Fig. 7.4 Fresh rice straw silage prepared with lactic acid bacteria inoculant

Chemical Composition and Feed Characteristics

Tea grounds are mainly by-products of readymade tea drinks, which are derived from tea leaves extracted by hot water. Tea leaf has a useful content of amino acids, proteins, vitamins, tannins, and polyphenols (Cai 2001; Xu et al. 2007). Most of these nutrients remain in the tea grounds after extraction to make tea drinks.

After extraction, green tea grounds (GTG) usually contain 22–35 % of CP, 2.3–7.1 % ether extract (EE), 26–37 % acid detergent fiber (ADF) and 37–45 % neutral detergent fiber (NDF) on DM basis (Cai 2001; Xu et al. 2007; Kondo et al. 2004). Barley tea grounds (BTG) contain 13–19 % CP, 2.2–3.4 % EE and 28–34 % NDF on DM basis (Xu et al. 2003). All these suggest that tea grounds have a potential as a feed resource.

However, the high moisture content makes it deteriorated easily after being released by beverage companies. Therefore, it must be used as soon as possible if fed directly. Whereas, direct-fed exhibits poor palatability due to the tannin components existed in tea grounds. Furthermore, drying process will consume a lot of energy if used as dry feed. Therefore, ensiling is one of the suitable ways to preserve high-moisture tea grounds.

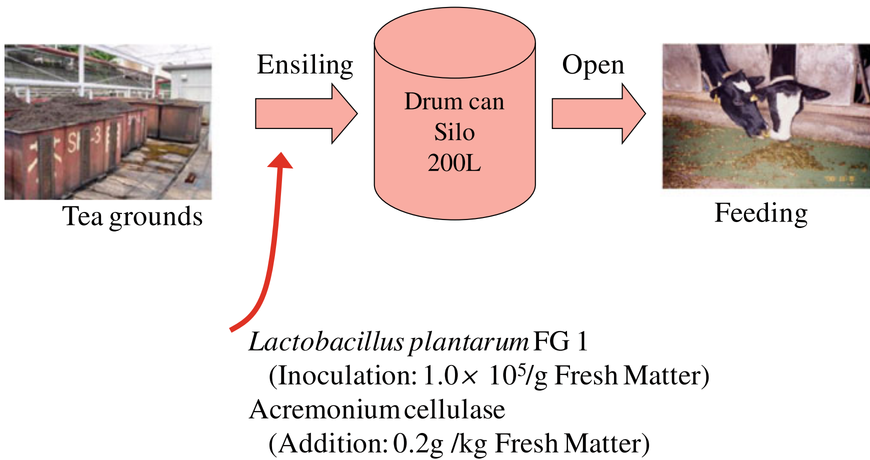


Fig. 7.5 Preparation method of tea grounds silage

Silage Preparation and Fermentation Quality

Preservation by ensiling is highly dependent on lactic acid fermentation. Whereas, tea grounds usually contain about 10^6 aerobic bacteria, 10^3–10^4 mould and yeast in cfu g^{-1} of FM, whereas LAB could not be detected (<10 cfu g^{-1}). Besides, water-soluble carbohydrates are consistently at or below the detectable level (Cai 2001; Cai et al. 2001; Xu et al. 2008). Thereby, lactic acid fermentation scarcely occurs when untreated tea grounds are ensiled alone.

Fermentation Quality of Tea Grounds Silage

As shown in Figs. 7.5 and 7.6, the preparation method of tea grounds silage with a high quality was developed (Cai 2001). Tea grounds are usually ensiled with additives or in mixed silage with other feed materials. The addition of *L. plantarum* FG1 and commercial acremonium cellulase (AUS) in tea grounds greatly improved the fermentation quality and retained the nutrient components (Table 7.4). BTG treated with mixtures of *L. plantarum* FG1 and AUS, formic acid, and sodium hydroxide (NaOH) were also well preserved as indicated by enhanced lactic acid fermentation or restricted fermentation of almost all micro-organisms (Xu et al. 2008).

In addition, it has been found that the preparation of mixed silage is effective for GTG to improve fermentation quality. GTG could be ensiled successfully without bacterial inoculants when mixed with materials containing sufficient sugars (Nishino et al. 2007). Besides forage silage, the addition of GTG could also enhance the lactic acid fermentation of by-products-mixed silage when there are insufficient materials for lactic acid production (Kondo et al. 2006). Researches

Green tea grounds silage

Barley tea grounds silage

Digestion trial

Cattle feeding

Fig. 7.6 Tea grounds silage and nutrient evaluation

indicated that neither GTG-associated LAB nor tea polyphenols account for the enhancement of lactic acid fermentation. It is probably that GTG supply some nutrients which are heat-stable and effective for LAB growth during silage fermentation (Kondo et al. 2004).

Fermentation Quality of Tea Grounds TMR Silage

Ensiling is suitable for preserving high moisture tea grounds. However, there are problems with the use of tea grounds as feed, such as nutritional imbalance, poor palatability and poor preservation (Xu et al. 2004). If ensiled with dry feeds as a total mixed ration (TMR), the risk of effluent production would be minimised and the time for mixing prior to feeding could be omitted. In addition, unpalatable by-products could be incorporated into TMR if their odors and flavors could be altered by silage fermentation.

It has been shown that the substitution of GTG for wet brewers' grains at different ratios of TMR could be well preserved with high-lactic acid content, low pH and NH_3–N content (Xu et al. 2007). Besides, TMR silages prepared with BTG at ratios of 10, 20 and 30 % DM were also well preserved (Xu et al. 2004).

Table 7.4 Fermentation quality, chemical composition, apparent digestibility, and nutrient content of green tea grounds silage

	FG1 + AUS	Control
Fermentation profile		
pH	3.49[c]	4.98[a]
Lactic acid (%FM)	1.55[a]	0.23[c]
Acetic acid (%FM)	0.52	0.50
Propionic acid (%FM)	ND	ND
Butyric acid (%FM)	ND	ND
Ammonia-N (%FM)	0.03	0.05
Chemical composition		
Dry matter (%)	25.2	25.5
Organic matter (DM%)	96.7	96.9
Crude protein (DM%)	31.3	31.0
Ether extract (DM%)	6.1	6.0
Acid detergent fiber (DM%)	21.5[b]	23.2[a]
Apparent digestibility		
Dry matter (%)	70.6 ± 2.8	–
Crude protein (%)	74.6 ± 4.4	–
Ether extract (%)	50.7 ± 2.9	–
Acid detergent fiber (%)	50.2 ± 2.2	–
Energy (%)	67.4 ± 1.9	–
Nutrient content		
Total digestible nutrients (DM%)	71.1 ± 0.3	–
Digestible energy (MJ/kg DM)	13.4 ± 0.4	–

FG1 Lactobacillus plantarum, *AUS Acremonium cellulase*
FM fresh matter, *DM* day matter
[a–c], [a, b] and [a, c] Values within the same row with different superscript letters differ significantly at $p < 0.05$ and $p < 0.01$, respectively

Nutritive Value of Tea Grounds Silage and Tea Grounds TMR Silage

Nutrient losses and proteolysis are inevitable during the ensiling process. They are always reduced or inhibited by a rapid decline in pH during the initial period to create a low pH environment that is unsuitable for the action of plant and microbial proteases. In addition to the fermentation characteristics of silage, their potential nutritive value for ruminants also need to be evaluated as assessed by FI, digestibility, nitrogen (N) balance, rumen fermentation, and blood component.

Nutritive value of tea grounds silage

Many researchers have studied the nutritive value of tea grounds and tea grounds silage so as to fully access the feeding effect of tea grounds silage treated with different additives or ensiled as mixed silage.

The nutritive value of GTG is thought to be equivalent to that of brewers' grains. Digestibilities of CP, EE and ADF for GTG silage are 74.6, 50.7 and 50.2 %, and the

estimated total digestible nutrients (TDN), digestible CP (DCP), and DE are 71.1, 23.9 % and 13.4 MJ/kg on DM basis, respectively (Xu et al. 2003).

However, digestibilities of CP, organic cell wall and ADF for BTG are 32.5, 28.8 and 21.2 %, and the estimated TDN, DCP, DE, and metabolisable energy are 71.0, 2.9 %, 12.9, and 10.7 MJ/kg on DM basis, respectively. It is suggested that the nutritive value of BTG silage is about 80 % of that of barley and they can be used as a potential feed ingredient (Xu et al. 2003). Xu et al. (2008) suggested that BTG silage treated with NaOH had the highest ruminal degradabilities of DM and CP, then it followed by LAB + AUS treatment. However, formic acid treatment had no effect on the DM and CP disappearance of silage (Xu et al. (2008).

Previous study indicated that ensiling may degrade part of the components of tea catechins, while not affecting total phenols, antioxidative activity and digestibility of GTG (Nishino et al. 2007). It is inconsistent with conclusions that tannins could prevent protein degradation during forage ensiling and studies that the addition of GTG could increase ruminal gas production and decrease the DM loss (Kondo et al. 2006).

Nishida et al. (2006) suggested that feeding diets containing 20 % of GTG silage on dietary DM had no negative impact on ruminal fermentation, but increased the plasma antioxidative activity and vitamin E concentration (Nishida et al. 2006).

Nutritive value of tea grounds TMR silage

GTG contain a lot of protein and functional components, such as tannin, caffeine, betacarotene and vitamin E (Cai 2001). Therefore, it can be used as an additive to improve low quality silage instead of the currently using expensive protein supplements. Previous study has indicated that condensed tannin (CT) at levels of 25–40 g/kg DM can protect amino acids to increase the absorption in the small intestine of ruminants, while deteriorating intake and digestibility at >55 g/kg DM (Min et al. 2003). All these suggest that many possible results will occur on the utilisation of tea grounds, and the proper addition of tea grounds need further study.

Many researches have investigated the feeding value of different application rates of tea grounds. Xu et al. (2004) found that with the increasing proportions of BTG in TMR silage, digestibilities of DM and CP, and TDN in TMR silages containing 10 and 20 % BTG were significantly higher than that of 30 % BTG (Xu et al. 2004). Therefore, the ideal mixing proportion of the BTG for TMR silage is 10–20 % in DM basis.

Progressive increases of GTG (0, 5, 10, and 15 % on DM basis) substituted for wet brewers grains had no effect on the voluntary FI of TMR silage, while digestibilities of DM, organic matter, CP, and energy were slightly lower than the control, and the N intake and fecal N increased. No differences among treatments were observed in urinary N and retention N, pH level and total VFA concentration. However, the rumen NH_3–N contents tended to decrease with the increasing of GTG. Therefore, the high GTG level of 15 % of the diet DM can be recommended for silage based TMR (Xu et al. 2007).

Ensiling is a suitable method to preserve tea grounds and it is also fit for other high-moisture agricultural or food by-products. Both ensiled with additives and mixed ensiled with other high quality feed materials can improve the fermentation quality. Furthermore, adjusting moisture with dry feeds as a TMR has been applied universally in recent years.

In addition, tea grounds can be used as protein supplement. Considering the disagreement in the effect of applying tea grounds, it is important to fully access and make full use of tea grounds to reach better feed effect. According to the above researches, the ideal mixing proportion of tea grounds for TMR is about 10–20 % or less on DM basis.

7.2.3.4 Application of Lactic Acid Bacteria for Vegetable Residues Silage Making

Usually, vegetable by-products contain high amounts of protein and fiber suitable as animal feed. Vegetable residues as cabbage, Chinese cabbage and lettuce contain abundant nutrients such as vitamins, minerals and vegetable fiber, and large quantities of these vegetables are produced annually in many countries, including Japan and China.

These vegetables residues are easily perishable because of their high moisture content. Technologies to create good quality animal feed from vegetable residues and to provide long-term storage of the resulting silage need to be developed. Using vegetable residue silage as an animal feed has proven economically viable, not only as a way of disposing of vegetable residues but also as a real alternative for feeding livestock in regions where vegetable residues are the main food by-product. LAB is naturally present on the surface of forage crops, grasses, and some vegetables. They are responsible for silage fermentation and also influence fermentation quality (Yang et al. 2010).

The screen, isolate, and identify the LAB from vegetable residues, with particular interest in species that are most likely to play an important role in the fermentation process. Isolates were identified at the molecular level using 16S rDNA sequence analysis. To use the vegetables residues effectively for animal feed, their chemical composition and silage fermentation characteristics were also studied (Yang et al. 2010).

The vegetable residues of cabbage, Chinese cabbage, and lettuce were collected from a local commercial vegetable factory (Fig. 7.7). Natural populations of LAB and silage fermentation of vegetable residue were studied. Fifty-two strains of LAB isolated from vegetable residues of cabbage, Chinese cabbage, and lettuce were identified and characterised. These vegetable residues contained a high level of CP (20.2–28.4 % of DM). Strains isolated from vegetable residue were identified as *L. plantarum*, *Lactococcus piscium*, *Lactococcus lactis*, *Leuconostoc citreum*, *Weissella soli*, and *Leuconostoc gelidium*, respectively. The prevalent LAB, predominantly homofermentative lactobacilli, and *L. plantarum* was the dominate members of the LAB population in three types of vegetable residues.

Fig. 7.7 Vegetable residues collected from local vegetable factory in Japan

These silages prepared by using a small-scale fermentation system were well preserved, with a low pH and relatively high content of lactate. The study suggests that the vegetable residues contain abundant LAB species and nutrients, and that they could be well preserved by making silage, which is a potential good vegetable protein source for livestock diets.

As shown in Table 7.5, these vegetable residues have relatively high water-soluble carbohydrate content with 15–20 % on DM basis and high number of epiphytic lactobacilli (10^4 cfu g^{-1} of FM). During the silage fermentation, the lactobacilli could produce sufficient lactic acid to reduce pH and inhibit the growth of harmful bacteria, and the resulting silage was of good quality. Therefore, the vegetable residues contained many species of LAB and abundant nutrition content. Based on the silage fermentation and chemical composition analysis, we have found that the vegetable residue can be well preserved by preparing a silage and it has good potential as a vegetable protein source for livestock diets.

When high-moisture food by-products are ensiled as fermented by-product mix with dry feeds, the risk of effluent production can be minimised, rumen function stabilised, and self-selection by animals avoided. The moisture of the vegetable residues was adjusted to prepare good quality silage, and their digestive

Table 7.5 Microbiological analysis (viable microorganisms in fresh matter, FM), chemical composition and silage quality of vegetable residues

Item	Cabbage	Chinese cabbage	Lettuce	SEM
Counts (cfu g^{-1} of FM)				
Lactic acid bacteria	4.4×10^4	4.7×10^2	5.0×10^3	
Bacilli	4.5×10^3	ND[a]	1.5×10^3	
Coliform bacteria	6.5×10^5	7.0×10^3	6.0×10^4	
Aerobic bacteria	3.7×10^6	2.5×10^7	3.7×10^6	
Molds	ND	ND	ND	
Yeasts	1.4×10^4	3.0×10^3	1.5×10^3	
Lactobacilli	3.5×10^4	3.0×10^3	1.0×10^3	
Lactococci	ND	2.0×10^3	ND	
Leuconostoc	8.2×10^3	ND	1.0×10^3	
Weissella	ND	ND	3.5×10^3	
Chemical composition (% of DM)				
Organic matter	88.7[b]	82.2[b]	87.3[b]	0.77
Crude protein	20.2[b]	28.4[b]	27.7[b]	0.66
Ether extracts	6.1[b]	3.4[b]	2.5[b]	0.18
Acid detergent fiber	29.9[b]	26.4[b]	21[b]	0.57
Neutral detergent fiber	31.40	28.40	30.70	0.78
Glucose	9.2[b]	4[b]	3.8[b]	0.11
Fructose	9.1[b]	9.4[b]	10.4[b]	0.36
Sucrose	2.2[b]	1.8[b]	0.9[b]	0.64
Silage quality				
Moisture content (%)	94.2[b]	97.1[b]	95.8[b]	0.35
pH	3.6[b]	3.9[b]	3.8[b]	0.01
Lactic acid (%DM)	24.5[b]	24.8[b]	14.8[b]	0.10
Acetic acid (%DM)	8.3[b]	6.9[b]	4.8[b]	0.07
Butyric acid (%DM)	ND	ND	ND	
Propionic acid (%DM)	ND	ND	ND	
Ammonia–N (%DM)	3.6[b]	9.0[b]	3.6[b]	0.05

Silage was stored for 60 days; data are the average of three vegetable residue samples

[a] Not detected

[b] Means within rows with different superscripts differ ($P < 0.05$)

characteristics were studied. The silage treated with drying beet pulp or LAB had a lower pH and a higher lactic acid content than the control silage. The vegetable residues had high nutritional content and high in vitro DM digestibility. Also, both the addition of a LAB inoculant and moisture adjustment with beet pulp improved the fermentation quality of the vegetable residue silages (Cao et al. 2011).

7.2.3.5 Fruit By-product Silage Prepared with Lactic Acid Bacteria

Fruits are necessities in our daily life, and the demand was increasing over time. At the time we made large amount of by-products and residues. It became an important problem to fully and effectively utilise it to develop feed production.

Banana [*Musa sapientum* Linn], pineapple [*Ananascomosus* (L.) Merr.] and papaya [*Chaenomelesspeciosa* (Sweet) Nakai] are the most famous tropical fruits in the world. However, the excess fruit produced by farmers and the fruit residues from food processing plants are usually treated as food waste or compost. These fruit residues contain a certain amount of protein and fiber, which is suitable as animal feed. But, these fruit residues are easily perishable because of their high-moisture content and produced in tropic area. Using fruit residue silage as an animal feed has proven economically viable, not only as a way of disposing of fruit residues but also as a real alternative for feeding livestock in regions where fruit residues are the main agricultural by-product (Fig. 7.8). Based on the research of Yang et al. (2012) banana residues contained certain content of CP about with 7.06 % on a DM basis, and ADF was about 46.12 % of DM (Table 7.6). These silages prepared with LAB were well preserved with a low pH. The study suggests that the banana stems and leaves can be well preserved by making silage and it is good potential available resources for livestock diets.

The residue from fruit processing enterprises such as canned fruit factory, beverage factory, fruit production processing factory etc., are pulp, stone, peel, are primarily apple pomace, grape pomace, and citrus pulp. Every ton of fruit processing will generate 400–500 kg pomace, which is very considerable amount of waste to be utilised. The use of pomace as feeds in other countries such as the United States and Canada have demonstrated significant economic and environmental benefits. Pomace, grape pomace, and citrus pulp have been developed as standard feed ingredients (feed for pigs, chickens, cattle) which are included in the feed composition tables issued by the state. However, pomace is yet put into practical usage, and even discarded as waste in China, which causes significant economic and environmental loss.

Pomace is rich in nutrients and large amounts of water, and is also rich in sugar, pectin and other substances. Fruit by-products are rich in nutrients, which provides favorable conditions for the growth of microorganisms. If it is not consumed in a short period of time by animals, it gets moldy and no longer qualified as animal feed. Fortunately, the silage production from fruit by-products is technically feasible.

Huang et al. (1993) has used the pineapple processing waste as a raw material for silage, and found that the pineapple pomace silage after 40–50 days had fragrant smell, soft texture. Its CP content (mass fraction) is 7.69 % and crude fiber is 29 %, which could replace the green feed such as elephant grass and sweet potato vines to reduce the cost of feed. Results of fresh citrus peel silage confirm that the by-products to be a nutritious raw material for silage and the quality of silage is of good. A variety of methods are suitable for this kind of raw material of green silage (Zhang et al. 2003).

Shen et al. (2012) studied the effects of different addition of LAB and pineapple peel on the quality of *Stylosanthes* silage and aerobic stability, the results showed that adding either of LAB or pineapple peel can significantly improve the fermentation quality of *Stylosanthes* silage, but the maximal the quality is achieved

Banana stems and leaves Pineapple peel Papaya peel

Fig. 7.8 Fruit by-product

Table 7.6 Chemical composition and silage fermentation of Brazil banana stems and leaves

Sample	DM (%)	CP (DM%)	CF (DM%)	ADF (DM%)	pH	Lactate (DM%)	Ammonia–N (DM%)
Stems and leaves	13.30	7.06	2.83	46.12	6.42	ND	ND
Silage	13.16	6.90	2.86	43.24	4.25	2.76	2.10

DM dry matter, *CP* crude protein, *CF* crude fat, *ADF* acid detergent fibre, *ND* not detected

with a mixture of the two, especially pineapple mixed with *L. plantarum*. Addition of pineapple peel or LAB, or both, maintains aerobic stability as the control.

Xiao et al. (2010) investigated the effect of three different strains of LAB on the nutrients of apple pomace silage. The study found that, compared to natural silage of apple pomace that did not have any strains, LAB can effectively reduce the pH of silage material, in which a large number of harmful microorganisms can not survive so that prolonged the retention time of the feed and improved the security of the feed, simultaneously preserved the nutritional content of the feed. Their study also showed that a reasonable ratio of LAB and yeast not only increased the CP content in the feed, but also improved the quality.

These studies have suggested that LAB is the core of the silage of fruit by-product and its growth plays a critical role in the success of the pomace silage. In a nutshell, with the development of science and technology, LAB in the application of fruit by-product resources will be more and more in-depth, and produce huge social and economic benefits.

7.2.3.6 Lactic Acid Bacteria and Methane Emission Mitigation from Livestock

In ruminants, fermentative digestion by ruminal microorganisms produces not only energy and protein for tissue metabolism but also methane, carbon dioxide, and ammonia (Fig. 7.9). Methane production is an energetically wasteful process, because the gas must be eructated from the rumen. The energy lost to methane production ranges from 2 to 12 % (Giger-Reverdin and Sauvant 2000).

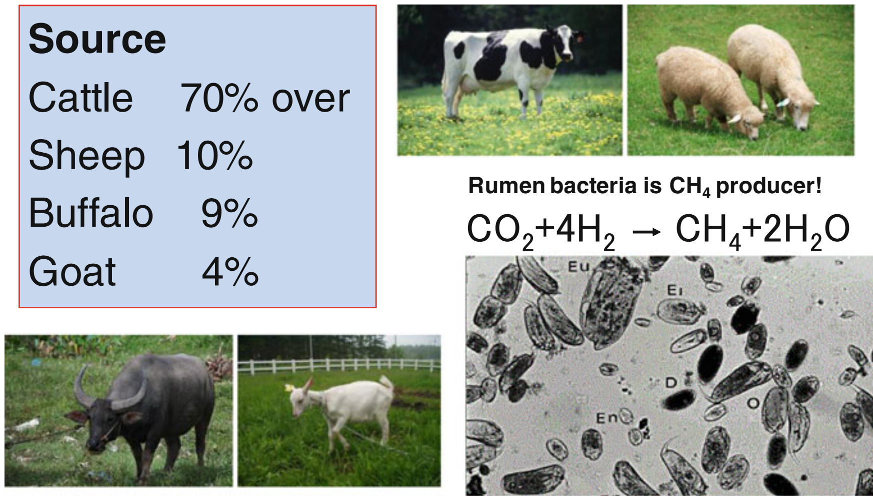


Fig. 7.9 Emission of CH_4 from ruminants contribute 15–19 % of the total emissions of CH_4

Furthermore, methane emission by ruminants is considered the single largest source of anthropogenic methane and the second most important greenhouse gas (Mathison et al. 1998). Consequently, it is necessary to understand the factors that influence enteric methane production. Doing so would not only reduce uncertainty about the contribution of methane to greenhouse gas emissions and help develop viable greenhouse gas reduction strategies, but also provide an economic benefit by leading to greater energy-used efficiency of the feed. There is increased worldwide interest in mitigating the impact of ruminant-produced methane.

Ruminants and so called pseudoruminants (e.g., Camelidae, some birds) have large anaerobic fermentation chambers at both ends of their digestive tract, which greatly facilitate the digestion of carbohydrates and plant cell walls. Furthermore, microbial proteins synthesised in the forestomach are also digested in the small intestine, and provide up to more than 50 % of the amino acids entering the blood stream (Moss et al. 2000).

The microbial ecosystem involved in propionate formation differs according to dietary conditions. The cellulolytic bacteria *Fibrobacter succinogenes* is the major propionate producer via the succinate pathway in animals fed roughage diets, while lactate is the main intermediate in the conversion of starch to propionate. LAB tolerate low pH conditions, and therefore are able to use H_2 and compete with methanogens even in unfavorable pH conditions.

Of the many alternative approaches to reducing methane, both in terms of reduction per animal and reduction per unit of animal product, the most promising areas are the development of new antimethanogenic compounds or alternative electron acceptors in the rumen and the reduction of protozoa in the rumen.

The most widely used microbial feed additives (live cells and growth media) are based on *SC* and *AO*. Their effects on rumen fermentation and animal

Table 7.7 Nutrient digestibility, daily energy intake and methane emission of sheep fed total mixed ration silage treated with LAB

	Treatment		SEM	*P*-value
	Control	FTMR		
Gross energy (GE) intake				
kJ	19559.2	19810.4	672.3	0.8058
kJ/kg of $BW^{0.75}$	1038.1	1045.3	1.0	0.6433
Methane emission				
L	39.84	29.84	0.82	0.0001
L/kg of DMI	39.87	30.03	1.29	0.0017
L/kg of DDM	60.87	43.61	2.10	0.0012
L/kg of $BW^{0.75}$	2.12	1.58	0.10	0.0007
Methane energy				
kJ	1576.4	1180.8	32.6	0.0001
kJ/kg of $BW^{0.75}$	83.8	62.5	2.3	0.0007
J/KJ of GE intake	80.8	59.9	2.6	0.0013
Apparent digestibility				
Dry matter	0.656	0.689	0.0094	0.0518
Organic matter	0.711	0.738	0.0084	0.0650
Crude protein	0.648	0.704	0.0075	0.0023
Ether extract	0.789	0.840	0.0114	0.0282
Nonfibrous carbohydrate	0.925	0.913	0.0086	0.4268
Acid detergent fibre	0.491	0.549	0.0186	0.0741
Neutral detergent fibre	0.586	0.655	0.0110	0.0043
Gross energy	0.709	0.742	0.0083	0.0334

FTMR fermented total mixed ration, *DMI* dry matter intake, *DDM* digestible dry matter

productivity are wide ranging (Martin 1998). However, very little information is available on their effects on methane production. Shu et al. (1999) showed that immunisation can successfully reduce the numbers of streptococci and lactobacilli in the rumen. Cao et al. (2010) determined the effect of LAB on fermentation quality and in vitro ruminal digestion of vegetable residue silage, and reported that LAB increased DM digestibility and decreased ruminal methane production (Table 7.7).

7.3 Summary and Perspective

In recent years, an acknowledgment has been made that the application of LAB inoculant and cellulase could improve the fermentation quality of silage greatly. The improvement of the bacterial community in the digestive tract and health of animals reveals the effect of LAB on silage storage and livestock production and has caught the attention of many scientists. In addition, the exploration of LAB with a high performance in silage making is noteworthy for future research and is

extremely important for developing safe livestock production and for increasing self-supplying feed. The use of roughage in livestock production and the push for self-supplying feed use are also very necessary for building a resource recycling society, for reducing the environmental burden, for utilising idle land efficiently, and for strengthening the utilisation of unused feed resources.

The exploitation of a new utilisation method for fermented feed LAB as a target, the study of taxonomy and the exploration of fermentation performance not only provide new insights into the study of the basic theory of the modulation of feed processing but also provide for the development and utilisation of new LAB inoculant preparation techniques, modulation technology for whole plant rice and crop straw roughage and storage fermentation technology for food residue. These results show how unused resources play an important role in increasing the feed self-sufficiency rate, reducing the environmental load and exploiting new research areas. In the grassland and animal husbandry areas, the examination of the effect of LAB from the front view, such as safe and secure production of livestock products, is especially important for exploring the unknown LAB and its potential function. In the future, the use of LAB will be a research hotspot and will provide an excellent defence for animal intestines to modulate high quality TMR with food by-products and crops residues and to develop technology for healthy livestock production.

References

Abe T, Mujahid A, Sato K, Akiba Y, Toyomizu M. Possible role of avian uncoupling protein in downregulating mitochondrial superoxide production in skeletal muscle of fasted chickens. FEBS Let. 2006;580:4815–22.

Anadón A, Martinez-Larranaga MR, Aranzazu Martínez M. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul Toxicol Pharm. 2006;45:91–5.

Barrington GM, Gay JM, Evermann JF. Biosecurity for neonatal gastrointestinal diseases. Vet Clin N Am: Food A. 2002;18:7–34.

Biavati B, Mattarelli P. The family Bifidobacteriaceae. In: Dworkin M, editor. The prokaryotes: an evolving electronic resource for the microbiological community, vol 3. New York: Springer; 2006. p. 322–82.

Buenrostro JL, Kratzer FH. Effects of *lactobacillus* inoculation and antibiotic feeding of chickens on availability on dietary biotin. Poult Sci. 1983;62:2022–9.

Cai Y. The role of lactic acid bacteria in the preparation of high fermentation quality. Grassland Sci. 2001;47:527–33.

Cai Y, Benno Y, Ogawa M, Ohmomo S, Kumai S, Nakase T. Influence of Lactobacillus spp. from an inoculant and of Weissella and Leuconostoc spp. from forage crops on silage fermentation. Appl Envir Microbio. 1998;64:2982–7.

Cai Y, Masuda N, Fujita Y, Kawamoto H, Ando S. Development of a new method for preparation and conservation of tea grounds silage. Anim Sci J. 2001;72:536–41.

Cai Y, Kawashima T, Mitsumoto M, Xu C, Saeki M. Microorganism preparation for feed and its utilization. 2007; Patent: P07A010622.

Cao Y, Takahashi T, Horiguchi K, Yoshida N, Cai Y. Methane emissions from sheep fed fermented or non-fermented total mixed ration containing whole-crop rice and rice bran. Anim Feed Sci Technol. 2010;157:72–8.

Cao Y, Cai Y, Takahashi T, Yoshida N, Tohno M, Uegaki R, Nonaka K, Terada F. Effect of lactic acid bacteria inoculant and beet pulp addition on fermentation characteristics and in vitro ruminal digestion of vegetable residue silage. J Dairy Sci. 2011;94:3902–12.

Castillo NA, LeBlanc AM, Galdeano CM, Perdigón G. Probiotics: an alternative strategy for combating salmonellosis immune mechanisms involved. Food Res Int. 2012;45:831–41.

Champagne CP, Roy D, Gardner N. Challenges in the addition of probiotic cultures to foods. Crit Rev Food Sci Nutr. 2005;45:61–84.

Chang C, Chen Y, Chiou M, Su C, Chen DS, Tsai C, Yu B, Hsu Y. Application of *Scutellariae radix*, *Gardeniae fructus*, and probiotics to prevent *Salmonella enterica* Serovar Choleraesuis infection in swine. Evid-Based Compl Alt. 2013. doi: 10.1155/2013/568528 .

Chaveerach P, Keuzenkamp DA, Lipman LJ, Van KF. Effect of organic acids in drinking water for young broilers on *Campylobacter* infection, volatile fatty acid production, gut microflora and histological cell changes. Poult Sci. 2004;83:330–4.

Chen CY, Tsen HY, Lin CL, Yu B, Chen CS. Oral administration of a combination of select lactic acid bacteria strains to reduce the Salmonella invasion and inflammation of broiler chicks. Poult Sci. 2012;91:2139–47.

Choi JY, Kim JS, Ingale SL, Kim KH, Shinde PL, Kwon IK, Chae BJ. Effect of potential multimicrobe probiotic product processed by high drying temperature and antibiotic on performance of weanling pigs. J Anim Sci. 2011;89:1795–804.

Church DC, Pond WG. The gastrointestinal tract and nutrition. In: Basic animal nutrition and feeding. Albany: Albany Printing; 1974. p. 25–49.

Eun JS, Beauchemin KA. Relationship between enzymic activities and in vitro degradation of alfalfa hay and corn silage. J Anim Feed Sci Technol. 2008;145:53–67.

Fajardo P, Pastrana L, Méndez J, Rodríguez I, Fuciños C, Guerra NP. Effects of Feeding of two potentially probiotic preparations from lactic acid bacteria on the performance and faecal microflora of broiler chickens. Sci World J. 2012. doi: 10.1100/2012/562635 .

Fisher K, Phillips C. The ecology, epidemiology and virulence of *Enterococcus*. Microbiol. 2009;155:1749–57.

Gabriela P, Roy F, Raúl R. Lactic acid bacteria and their effect on the immune system. Curr Issues Int Microbiol. 2011;1:27–42.

Giang HH, Viet TO, Ogle B, Lindberg JE. Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. Livest Sci. 2010;129:95–103.

Giger-Reverdin S, Sauvant D. Methane production in sheep in relation to concentrate feed compostion from bibliographic data. In: Ledin I, Morand-Fehr P, editors. 8th seminar of the sub-network on nutrition of the FAO-CIHEAM inter-regional cooperative research and development network on sheep and goats. France: INRA, Cahiers-Options-Mediterraneennes, Grignon; 2000. p. 43–6.

Havenitha CEG, Seegersa JFML, Pouwels HP. Gut-associated lactobacilli for oral immunization. Food Res Int. 2002;35:151–63.

Huang L, Liao J, Lai X. Pineapple peel fed cattle technology exploitation. Chin J Anim Sci. 1993;29:13–4.

Joint FAO/WHO Expert Committee. Monographs Prepared by the Fifty-eighth Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Rome; 2002. p. 21–7.

Julio V, Maria LS, Oliveira PCD, Ferreira SS, Susana A. Lactic acid bacteria in the prevention of pneumococcal respiratory infection: future opportunities and challenges. Int Immunopharmacol. 2011;15:1633–45.

Kondo M, Naoki N, Kazumi K, Yokota H. Enhanced lactic acid fermentation of silage by the addition of green tea waste. J Sci Food Agr. 2004;84:728–34.

Kondo M, Kita K, Yokota HO. Evaluation of fermentation characteristics and nutritive value of green tea waste ensiled with by-products mixture for ruminants. Asian Australas J Anim Sci. 2006;19:533–40.

Leavis HL, Bonten MJM, Willems RJL. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. J Curr Opin Microbiol. 2006;9:454–60.

Lee NK, Choi IA, Park YH, Kim JM, Kim JM, Jung SC, Paik HD. Screening of antimicrobial lactic acid bacteria against bovine mastitis. Korean J Food Sci Ani Resour. 2007;27:543–7.

Lin D. Inhibition of the livestock and poultry intestinal disease by the lactic acid bacteria. Feed Husbandry. 1993;4:12–3.

Maldonado GC, LeBlanc AM, Vinderola G, Bibas BME, Perdigón G. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clin Vaccine Immunol. 2007;14:5485–92.

Martin SA. Manipulation of ruminal fermentation with organic acids: review. J Anim Sci. 1998;76:3123–32.

Matar C, Amiot J, Savoie L, Goulet J. The effect of milk fermentation by *Lactobacillus helveticus* on the release of peptides during in vitro digestion. J Dairy Sci. 1996;79:971–9.

Mathison GW, Okine EK, McAllister TA, Dong Y, Galbraith J, Dmytruk OIN. Reducing methane emissions from ruminant animals. J Appl Animal Res. 1998;14:1–28.

Matsuzaki T, Chin J. Modulating immune responses with probiotic bacteria. Immunol Cell Biol. 2000;78:67–73.

Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008;453:620–5.

McDonald P, Henderson AR, Heron SJE. The biochemistry of silage. Marlow: Chalcombe Publications; 1991.

Menconi A, Wolfenden AD, Shivaramaiah S, Terraes JC, Urbano T, Kuttel J, Kremer C, Hargis BM, Tellez G. Effect of lactic acid bacteria probiotic culture for the treatment of *Salmonella enterica* serovar Heidelberg in neonatal broiler chickens and turkey poults. Poult Sci. 2011;90:561–5.

Millemann Y. Diagnosis of neonatal calf diarrhoea. Rev Med Vet. 2009;160:404–9.

Min B, Barry T, Attwood G, McNabb W. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim Feed Sci Technol. 2003;106:3–19.

Modesto M, D'Aimmo MR, Stefanini I, Trevisi P, De Filippi S, Casini L, Mazzoni M, Bosi P, Biavati B. A novel strategy to select *bifidobacterium* strains and prebiotics as natural growth promoters in newly weaned pigs. Livest Sci. 2009;122:248–58.

Mori K, Ito T, Miyamoto H, Ozawa M, Wada S, Kumagai Y, Matsumoto J, Naito R, Nakamura S, Kodama H, Kurihara Y. Oral administration of multispecies microbial supplements to sows influences the composition of gut microbiota and fecal organic acids in their post-weaned piglets. J Biosci Bioeng. 2011;112:145–50.

Moss AR, Jouany JP, Newbold J. Methane production by ruminants: its contribution to global warming. Ann Zootech. 2000;49:231–53.

Nishida T, Eruden B, Hosoda K, Matsuyama H, Nakagawa K, Miyazawa T, Shioya S. Effects of green tea (*Camellia sinensis*) waste silage and polyethylene glycol on ruminal fermentation and blood components in cattle. Asian Australas J Anim Sci. 2006;19:1728–36.

Nishino N, Kawai T, Kondo M. Changes during ensilage in fermentation products, tea catechins, antioxidative activity and in vitro gas production of green tea waste stored with or without dried beet pulp. J Sci Food Agr. 2007;87:1639–44.

Orpin CG, Joblin KN. The rumen anaerobic fungi. In: The rumen microbial ecosystem. Hobson PN, Stewart CS, Hobson PN, Stewart CS, editors. London: Chapman and Hall; 1997. p. 140–95.

Pang H, Zhang M, Qin G, Tan Z, Cai Y. Identification of lactic acid bacteria isolated from corn stovers. Anim Sci J. 2011;82:642–53.

Pieper R, Janczyk P, Urubschurov V, Hou Z, Korn U, Pieper B, Souffrant WB. Effect of *Lactobacillus plantarum* on intestinal microbial community composition and response to enterotoxigenic *Escherichia coli* challenge in weaning piglets. Livest Sci. 2010;133:98–100.

Rinkinen M, Matto J, Salminen S, Westermarck E., Ouwehand AC. In vitro adhesion of lactic acid bacteria to canine small intestinal mucus. J Anim Physiol Anim Nutr. 2000;84:43-7.

Rokka T, Syvaoja EL, Tuominen J, Korhonen H. Release of bioactive peptides by enzymatic proteolysis of *Lactobacillus* GG fermented UHT milk. Milchwissenschaft. 1997;52:675–8.

Rosenquist H, Nielsen NL, Sommer HM, Nørrung B, Christensen BB. Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. Int J Food Microbiol. 2003;83:87–103.

Ross GR, Gusils C, Oliszewski R, Holgado SC, González SN. Effects of probiotic administration in swine. J Biosci Bioeng. 2010;109:545–9.

Shen C, Chen M, Li G, Zhang J. Add *lactobacillus* and pineapple effect on stylo silage quality. Acta Prataculutral Sci. 2012;21:192–7.

Shu Q, Gill HS, Hennessy DW, Leng RA, Bird SH, Rowe JB. Immunisation against lactic acidosis in cattle. Res Vet Sci. 1999;6:65–71.

Siggers RH, Siggers J, Boye M, Thymann T, Mølbak L, Leser T, Jensen BB, Sangild PT. Early administration of probiotics alters bacterial colonization and limits diet-induced gut dysfunction and severity of necrotizing enterocolitis in preterm pigs. J Nut. 2008;138:1437–44.

Signorini ML, Soto LP, Zbrun MV, Sequeira GJ, Rosmini MR, Frizzo LS. Impact of probiotic administration on the health and fecal microbiota of young calves: a meta-analysis of randomized controlled trials of lactic acid bacteria. Res Vet Sci. 2012;93:250–8.

Stern NJ, Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP, Pokhilenko VD, Levchuk VP, Svetoch OE, Seal BS. Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. Antimicrob Agents Chemother. 2006;50:3111–6.

Suo C, Yin Y, Wang X, Lou X, Song D, Wang X, Gu Q. Effects of *Lactobacillus plantarum* ZJ316 on pig growth and pork quality. BMC Vet Res. 2012;. doi: 10.1186/1746-6148-8-89 .

Sutas Y, Hurme M, Isolauri E. Down-regulation of anti-CD3 antibody-induced IL-4 production by bovine caseins hydrolysed with *Lactobacillus* GG-derived enzymes. Scand J Immunol. 1996;43:687–9.

Taheri HR, Moravej H, Tabandeh F, Zaghari M, Shivazad M. Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. Poult Sci. 2009;88:1586–93.

Timmerman HM, Mulder L, Everts H, van Espen DC, van der Wal E, Klaassen G, Rouwers SMG, Hartemink R, Rombouts FM, Beynen AC. Health and growth of veal calves fed milk replacers with or without probiotics. J Dairy Sci. 2005;88:2154–65.

Timms L. Observations of the bacterial flora of the alimentary tract in three groups of normal chickens. Br Vet J. 1968;124:470–8.

Trevisi P, De Filippi S, Minieri L, Mazzoni M, Modesto M, Biavati B, Bosi P. Effect of fructooligosaccharides and different doses of *Bifidobacterium animalis* in a weaning diet on bacterial translocation and Toll-like receptor gene expression in pigs. Nutr Abstr Rev. 2008;24:1023–9.

Trevisi P, Casini L, Coloretti F, Mazzoni M, Merialdi G, Bosi P. Dietary addition of *Lactobacillus rhamnosus* GG impairs the health of *Escherichia coli* F4-challenged piglets. Anim. 2011;9:1354–60.

Vanbelle M, Bertin G, Hellings P. Publication No. 39 of the nutritional biochemistry laboratory, Louvain-la-Neuve. 1985.

Walsh MC, Rostagno MH, Gardiner GE, Sutton AL, Richert BT, Radcliffe JS. Controlling Salmonella infection in weanling pigs through water delivery of direct-fed microbials or organic acids. Part I: effects on growth performance, microbial populations, and immune status. J Anim Sci. 2012;90:261–71.

Watkins BA, Kratzer FH. Effect of oral dosing of Lacrobacillus strains on gut colonization and liver biotin in broiler chicks. Poult Sci. 1983a;62:2088–94.

Watkins BA, Kratzer FH. Drinking water treatment with a commercial preparation of a concentrated Lacrobacillus culture for broiler chickens. Poult Sci. 1983b;63:1671–3.

Whittenbury GW. An investigation of the lactic acid bacteria. Ph.D. Thesis, University of Edinburg. 1961.
Xiao J, Lai H, Jiang L, Xue Q, Zhang H, Wu C. Influence of different strains on the efficiency of silage apple pomace fermentation. J Northwest A&F Univ. 2010;38:83–8.
Xu C, Cai Y, Fujita Y, Kawamoto H, Sato T, Masuda N. Silage preparation of barley tea grounds and their nutritive value. Nihon Chikusan Gakkaiho. 2003;74:343–8.
Xu C, Cai Y, Murai M. Fermentation quality and nutritive value of total mixed ration silage with barley tea grounds. Nihon Chikusan Gakkaiho. 2004;75:185–91.
Xu C, Cai Y, Moriya N, Ogawa M. Nutritive value for ruminants of green tea grounds as a replacement of brewers' grains in totally mixed ration silage. Anim Feed Sci Technol. 2007;138:228–38.
Xu C, Cai Y, Zhang H, Fukasawa M, Moriya N. Ensiling and subsequent ruminal degradation characteristics of barley tea grounds treated with contrasting additives. Anim Feed Sci Technol. 2008;141:368–74.
Yang JB. The application of lactic acid bacteria in medicine. In: Yang JB, Fu XL, editors. Lactic acid bacteria: biological basis and application. Beijing: China Light Industry Press; 1999. p. 178–82.
Yang JS, Cao Y, Cai Y, Terada F. Natural populations of lactic acid bacteria isolated from vegetable residues and silage fermentation. J Dairy Sci. 2010;93:3136–45.
Yang JS, Zhou H, Wang D, Tan H, Cai Y. Study on microbial flora's change and fermentation quality in ensiling of banana stems and leaves. Adv Mater Res. 2012;524:2316–20.
Yoshida Y, Tsukahara T, Ushida K. Oral administration of *Lactobacillus plantarum* Lq80 and *Megasphaera elsdenii* iNP-001 induces efficient recovery from mucosal atrophy in the small and the large intestines of weaning piglet. Anim Sci J. 2009;80:709–15.
Zhang JG. Roles of biological additives in silage production and utilization. Res Adv Food Sci. 2002;3:37–46.
Zhang S, Zhou H. The mode of fresh citrus peel pomace silage and quality assessment of the feed. Anim Sci Vet Med. 2003;20:28–9.
Zhang JG, Cai Y, Kobayashi R, Kumai S. Characteristics of lactic acid bacteria isolated from forage crops and their effects on silage fermentation. J Sci Food Agr. 2000;80:1455–60.

Chapter 8
Traditional Chinese Fermented Dairy Foods

Heping Zhang, Xia Chen, Tong Dan and Jie Dong

Abstract China is a multinationality country composed of various ethnic minority groups. The Chinese food culture is famous worldwide, especially traditional fermented dairy products. Many ethnic minority groups currently residing in provinces and areas, including Inner Mongolia, Xinjiang, Tibet, Chuanxi Plateau, Qinghai, still use ancient and traditional methods for fermented dairy product processing (such as yoghurt, koumiss, cheese-like products). There are abundant LAB resources in these traditional dairy products, which can well preserve the precious benefit bacteria from generation to generation. The principal raw material of the traditional dairy products is raw milk, which is mainly processed by natural fermentation. Traditional dairy products have high nutritional value. Besides, they also exert health-related probiotic benefits to human beings. In this chapter, we introduce the history, processing procedure, nutritional value and microbial biodiversity of traditional fermented dairy products.

Keywords Traditional fermented dairy products · History · Procedure · Nutritional value · Microbial biodiversity

H. Zhang (✉) · X. Chen · T. Dan
Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education,
Inner Mongolia Agricultural University, Hohhot, China
e-mail: hepingdd@vip.sina.com

X. Chen
e-mail: chenxia8280@163.com

T. Dan
e-mail: dantong813218@aliyun.com

J. Dong
Institute for the History of Science, Inner Mongolia Normal University, Hohhot,
People's Republic of China
e-mail: djnmdongjie@126.com

H. Zhang and Y. Cai (eds.), *Lactic Acid Bacteria*,
DOI: 10.1007/978-94-017-8841-0_8,
© Springer Science+Business Media Dordrecht 2014

8.1 History

The production techniques of Chinese fermented dairy products have a long history. In ancient times, fermented cow and mare milk products were most common. Moreover, goat milk was also used as raw material for traditional fermented milk products.

8.1.1 Early Chinese Fermented Dairy Products

In ancient times in China, fermented dairy products were generally known as 'Lao' ('Lao' is Chinese style fermented milk), fermented cow milk was known as 'cow milk Lao', fermented mare milk as 'mare milk Lao' and fermented goat milk as 'goat milk Lao'. The dairy products in the Central Plains of China and their production techniques mainly originated from the nomadic tribes of Northern China. The Huns were a nomadic tribe living in desert areas in Mongolia and the grasslands in Northern China, and nomadism was their basic lifestyle. They were used to eating meat and drinking milk, and although they occasionally ate the foods of the Han nationality, they usually discarded them because these foods did not cater to their taste and preference to 'Dong Lao'. The Huns considered 'Dong Lao' a delicacy. 'Dong' and 'Lao' mean fermented horse and cow milk products. At that time, some of the Huns became officials of the Han Dynasty, and they offered the koumiss brewing method to the imperial court. The koumiss brewing method of the Huns is as follows: first, the starters were placed into a mare milk containing leather pouch, but it is estimated that the most common practice was to add fresh mare milk into fermented mare milk. It was then pounded and mixed continuously to enable the lactic acid bacteria (LAB) to ferment and brew koumiss. In addition to the leather pouch, the Huns also used a kind of pottery, formerly known as 'Fu Ni', to hold mare milk. Besides the Huns, the nomadic tribes once living on the grasslands of Northern China, including Wuhuan, Siberians, Gaoche, Turkut, Khitans, also inherited the fermented dairy production technology.

In ancient times in China, the main diet of the people of the Central Plains was unlike those of the northern nomadic tribes and were not dairy-based, although they did also consume milk and fermented dairy products. *A New Account of Tales of the World* (*Shi Shuo Xin Yu*) records that Lu Yuan, a supreme government official in charge of military affairs in East Jin Dynasty visited the Prime Minister Wang Dao, and Wang Dao served Lu Yuan, 'Lao'. Lu Yuan usually did not eat dairy products, so he got sick after returning home due to psychological discomfort. This shows that people at that time still had a bias towards dairy products. *Humor* (*Xiao Lin*) also recorded that the people from the State of Wu went to Beijing and were served 'Lao', and they vomited after returning home. However, *Essential Skill to Benefit the People* (*Qi Min Yao Shu*) by Jia Sixie of the Northern Wei Dynasty recorded some kinds of fermented dairy products and their

production techniques. The book gives a detailed description of the production techniques for 'Gan Lao', 'Lu Lao' (different types of 'Lao') and the production methods for the fermentation agents. The production technique for 'Lao' stated in *Essential Skill to Benefit the People* is basically the same as the modern production process for yoghurt. This means that as early as 1,400 years ago, the working class in Northern China had already systematically mastered the production technique for yoghurt (Zhang 1994).

8.1.2 Fermented Dairy Products in the Mongolian Empire Period

Around the ninth century, the outer Mongolian Plateau where the Turkut nomadic tribe could be found everywhere began the historical process of Mongolisation (Yi 1979). During more than 30 years of 1195–1227, Genghis Khan rose to power from Northern Mongolia, conquered the tribes in eastern Inner Mongolia, unified the Mongolian Plateau and established the Mongol Khanate (Cao 2007). Peng Daya, an envoy in the Southern Song Dynasty, recorded in *Short Notes on the Black Tartars* (*Hei Da Shi Lue*) that the Mongols collected the drawn mare milk in a leather container and had kept it for several days until it got slightly sour before drinking. The fermented milk was called koumiss. At that time, it had been common practice for the Mongols to brew koumiss. Xu Ting also witnessed how herdsmen brewed koumiss, and he inspected the technique for brewing black mare milk for Mongolian nobles. The French missionary Guillaumede Rubru-quis (about 1215–1270) gave the most detailed description of the production process and categories of Mongolian fermented dairy products of the early thirteenth century. He recorded the entire production process for Mongolian mare milk and especially mentioned that the artisans of the Mongol Empire could make use of siphon technology to suck koumiss. Guillaumede Rubru-quis also recorded how the Mongols made the fermented dairy product—grut, which was similar to the process for making 'Gan Lao' (Mongolian style dry cheese) stated in *Essential Skill to Benefit the People*. The process for making 'Lu Lao' is similar to the process for making the milk curd that is still a regular food for the Mongolian people today.

The Mongols usually made semi-solid fermented dairy products. Section 145 of *The Secret History of Mongols* (*Meng Gu Mi Shi*) describes that when the neck veins of Genghis Khan were injured in the battlefield, Lemie sneaked into the enemy camp and found a vat of 'Ta She La Hei' and served it to Genghis Khan. 'Ta She La Hei' was translated as 'Nai Lao' by Yu Dajun (2001). Jagchid (1979) considered it as 'Lao', i.e. a kind of semi-liquid food produced from slightly fermented milk, like yoghurt.

The nomadic tribes like Mongolian people made dairy products by fermenting and turning liquid milk into solid dairy products. On the one hand, solid diary

products are suitable for nomadic life, on the other hand, they can be kept for a longer time to increase food reserves. This can be considered as the curing technique for dairy products. Before the thirteenth century, the Mongols apparently had mastered this basic dairy processing technique.

8.1.3 Fermented Dairy Products in the Yuan Dynasty

In 1260, Genghis Khan's grandson Kublai Khan ascended the throne in Kaiping and then conquered the Central Plains and established the Yuan Dynasty. The establishment of the unitary state on the one hand promoted the Mongols to come into contact with the advanced culture of the Central Plains, and on the other hand pushed forward the development of the nomadic culture with the help of a series of inherent and complete feudal autocratic system of the empire in the Central Plains and affected the whole country. The establishment of the Yuan Dynasty facilitated the development of ancient Chinese dairy products and pushed it to a peak.

From the progenitor of the Yuan Dynasty to the 29th year of the Yuan Dynasty, the government of the Yuan Dynasty ordered to add 'Lao and Dong' in the list of monthly sacrifices as a recommendation. These dairy products were precious sacrifices in major sacrificial activities and were carefully fermented and selected by a professional sector. The official responsible for preparing sacrificial mare milk in the Yuan Dynasty was called 'Tai Pu Qing'. After Wuzong Khaishan became the emperor, he promoted 'Tai Pu Yuan', who was responsible for the management of horses to Attendant Second Rank, in November of the 11th year of Dade (Song 1978). The rank of 'Tai Pu Yuan' reached a historical peak. There was a special group of people called 'Kipchaks' in the Yuan Dynasty, who were responsible for brewing koumiss for the royal family of the Yuan Dynasty. *The History of the Yuan Dynasty • The Biography of Tu Tu Ha* (*Yuan Shi • Tu Tu Ha Zhuan*) states that the Kipchak served the emperor and managed horses, and the mare milk they offered was light in colour, delicious and was called black mare milk.

When the emperor and his courtiers gathered together for drinking at the palace feasts in the Yuan Dynasty, a large quantity of mare milk was always needed. When Xianzong ascended the throne, a banquet that lasted three days was held, at which 300 horses and cows, 5,000 sheep and 2,000 carriages of wine were served. Yuan Jue and Zhou Boqi wrote a poem to praise the rich food and wine at the banquet (Yuan 2005). Other fermented dairy products such as cow milk crisps were also essential at the banquets in the Yuan Dynasty. *Principles of Correct Diet* (*Yin Shan Zheng Yao*) depicted that: 'The float condensate in cow's milk is collected and boiled into crisps'. This shows that cow milk crisps were ghee extracted from fermented cow milk. In addition to cow milk crisps, 'Ti Hu' (similar to refined cream cheese) was also served at large banquets. *Principles of Correct Diet* recorded that: 'Take around 1,000 kg best quality ghee, boil and filter it and store it with a large ceramic urn. In winter, take the unfrozen ghee at the center of the

urn, and this is called Ti Hu'. Thus, it can be seen that 'Ti Hu' is a kind of fermented dairy product made from cow milk crisps after further refining. Yelu Zhu mentioned in *Poems of Eight Delicacies in Military Camps* (*Xing Zhang Ba Zhen Shi*) that 'Ti Hu' was the top one in the eight delicacies in military camps and a favourite of the Mongolian emperors (Zhuang 2005).

In addition, *Essentials of Agriculture, Sericulture, Clothing and Food* (*Nong Sang Yi Shi Cuo Yao*) by Lu Mingshan in the Yuan Dynasty describes a method and the mechanical devices used for making ghee. Thus it can be seen that the production process for cream in the Yuan Dynasty had developed from the manual process to the semi-mechanical process. The production principles of all the devices for making cream are similar to those of modern cream mixers, which is the most up to date ghee making process in ancient China.

8.1.4 Fermented Dairy Products in the Ming and Qing Dynasties

After the establishment of the Ming Dynasty, the Mongols retreated to the great desert in Northern China, and the consumption peak of dairy products in the Yuan Dynasty also disappeared gradually. However, unlike the earlier Siberians and Khitans, the Mongols still maintain their unique nomadic way of living, and dairy products are still the main components in their food.

The fermented milk wine brewing technique has been retained very well. *Yi Yu* by Miner Shanren of the Ming Dynasty, *The Customs of the Northern Nationalities* (*Bei Lu Feng Su*) by Xiao Daheng and other works have recorded the brewing processes for fermented milk wine in the Mongolian areas (Mine Shanren 1937; Xiao 1985). In the Qing Dynasty, some books in the Central Plains describe the koumiss brewing processes of the Mongols. Shen Tao once called the fermented koumiss beyond the Great Wall as 'Yan Bei Ba Zhen' (one of the eight delicacies of Yan Bei), with a slightly sweet taste and fragrance, which was often documented in official travel notes.

Relatively speaking, the consumption of dairy products in the Central Plains reduced significantly. In the Ming Dynasty, dairy products were rare. Zheng Xiaoren once read in an old file that if an imperial concubine had mutton in her day's food, then she would not ask for milk any more (Li 1986). Xie Zhaozhe stated in Investigations on the Five Categories of Things (*Wu Za Zu*) that at that time, wealthy families were making every effort to acquire the rare ingredients, including fermented mare milk (Xie 2001). Therefore, when introducing Chinese products to the Europeans in the late Ming Dynasty, Matteo Ricci said: 'Chinese people only drink cow milk and they do not use goat milk to make 'Nai Lao' or as drink (Matteo and Nicolas 1983)'. What Matteo Ricci said was inevitably biased, but it reflected to a certain extent the fact that consumption of diary products was at a low level in the Ming Dynasty. This was because the people in the Central

Plains of the Ming Dynasty knew little about the diary products beyond the Great Wall, and that some misunderstanding appeared. For example, Xie Zhaozhe said that koumiss in Northern China contained no yeast and malt and was made naturally. On the other hand, some people in the Central Plains still liked the fermented dairy products beyond the Great Wall after personally tasting them. For example, Volume 6 of Miscellaneous Notes from the Bamboo Leaf Pavilion (*Zhu Ye Ting Za Ji*) by Yao Yuanzhi of the Qing Dynasty stated that, he got drunk each time he drank koumiss (Yao 1982).

However, some regions in the Central Plains had made contributions to the progress of the production process of fermented cow milk products. There was a kind of fermented cow milk product called 'Bao Luo' in the Central Plains of the Ming and Qing Dynasties. *Dream Reminiscences of Tao An* (*Tao An Meng Yi*) by Zhang Dai recorded the shape and production process of 'Bao Luo'. In the production process of 'Bao Luo' (Zhang 2008), in order to allow milk fermentation, 'the milk was obtained and placed in a pot at night' until 'milk froth was around 1/3 m', which was the state of milk after fermentation. This type of fermented dairy products existed even in the late Ming Dynasty and the Qing Dynasty. Both *Brief Knowledge of Things* (*Wu Li Xiao Zhi*) by Fang Yizhi in the late Ming Dynasty and *Categorised anthology of petty matters from the Qing period* (*Qing Bai Lei Chao*) in Qing Dynasty included similar records on fermented dairy products.

8.1.5 Conclusion

Initially, traditional fermented dairy products in ancient China were strongly affected by the nomadic tribes in Northern China, and their development processes branched into the two nomadic evolution tracks of the Central Plains and the border nomadic tribe areas. The development of dairy products in the Central Plains was related to the establishment and strengthening of the nomadic regimes, and was associated with the several 'Hu-shi' (food of 'Hu', non-Han nationalities living in the north and west in ancient times) waves in the Chinese dietary history (Wang 2009). The dairy product fermentation techniques in the Central Plains tended to reduce the lactose ingredient in dairy products, making it better accepted by people. In addition, the people in the Central Plains paid more attention to high value-added dairy products, such as 'milk crisps' and 'Ti Hu', which were produced from the fermented dairy products, 'Lao'. For the nomadic tribes in border areas in China, dairy products are their important dietary sources. Nomadic tribes turn perishable liquid milk into solid fermented dairy products for its ease in storage and convenience in migration. These are the most important purposes for the creation and heritage of fermentation techniques.

8.2 Processing Technology

In China, the manufacture of a variety of dairy products has a long history that goes back to thousands of years. The nomadic minorities in China (such as Huns, Mongolians, Manchus, Hazakhs, Tibetans, Daurs, Ewenkis, Xizangs, Uygurs, Bais) live nomadic lives and are known for 'Eating animal meat, drinking animal milk, wearing leather and fur, and living in felt-made tents'. Moreover, dairy products have played an important role in their traditional lives. The traditional dairy products made have a number of properties including a long history and very particular manufacturing procedures and flavours. They are considered as an essential food for the nomadic minorities but are also popular foods for other Chinese people. The main types of traditional fermented dairy products include fermented milk products, cheese-like products and ghee; these foods have a high nutritional value (Hati et al. 2013). This traditional processing procedure can be considered as a good source for modern dairy products.

8.2.1 Processing Methods for Fermented Milk Products

Traditional fermented milk products use cow milk, goat milk, yak milk, mare milk or camel milk as the raw material. The basic processing principles and procedures are similar amongst the different minority groups, and they all use natural fermentation.

8.2.1.1 Yoghurt

Chinese traditional yoghurt can be divided into set yoghurt, which is firm, and stirred yoghurt, which is more liquid in texture. Based on the acidity of the yoghurt, it can be divided into Eedsen Su, Tarag and Airag (Sun et al. 2010b). Based on the fat ratio, it can be divided into skimmed yoghurt, low-fat yoghurt and high-fat yoghurt.

Procedure for Eedsen Su

Fresh cow milk is filtered through a cloth into a pot and then allowed to naturally ferment either indoors or in a yurt (Fig. 8.1). The fermentation temperature is approximately 20 °C and fermentation lasts for 1–2 days in summer and 3–4 days in winter. Removing the cream that forms a surface layer, leaves an underlayer of homogenised, solidified but soft, tofu-like white skimmed plain yoghurt, or Eedsen Su, with a final pH between 4.4 and 4.5. This set plain yoghurt from natural fermentation is very delicious; it not only has a yoghurt aroma but also low acidity

Fig. 8.1 Set fermentation of yoghurt

so both adults and children enjoy it. Eedsen Su can be eaten directly or used to prepare milk tofu and/or Eejig.

Procedure for Tarag

After making wurum, there is a lot of skimmed milk left over that can be used to make stirred yoghurt. The skimmed milk is heated to 40–45 °C, and then a starter culture reserved from previous yoghurt production is added at a ratio of 1:20–1:30 and stirred to mix evenly. The temperature of the skimmed milk will gradually fall and it will begin to coagulate within 30 min. After fermenting for about 3–4 h, the skimmed milk will have fully coagulated to form a soft tofu-like substance called Tarag. Fermentation continues until the following morning (about 20 h) by which time the pH will have dropped to between 4.0 and 4.5. Tarag can be eaten directly or further processed into fermented milk curd or dried milk. Traditionally, nomadic people always take 500 mL of the skimmed milk following coagulation in a small cloth bag, remove the water and then use it as the starter for the next fermentation. So, the starter for Tarag is a kind of delicate, lubricated, homogenised acid curd from the previous preparation with a low water content and pH 4.5.

Procedure for Airag

Airag is also a kind of stirred yoghurt with high acidity and fat content. Any unused fresh milk, whole-fat milk, skimmed milk and/or fresh milk mixed with concentrated milk from wurum production is poured into a specially designed barrel (wooden or earthenware) for natural fermentation to occur. The barrel is largely made from wood and is around 1 m in height. It has a narrow opening at the top (25 cm diameter) with a wooden cover and a wider base. Inside the barrel, there is a stirring stick with a paddle at the end (Fig. 8.2). The fermentation temperature required is around 25 °C and to maintain this the barrel is insulated with an outer cover made of felt or cotton. The final pH of the Airag produced is usually around 3.6. To obtain good Airag, it needs to be stirred frequently

Fig. 8.2 The wooden barrel used for stirred yoghurt

Fig. 8.3 Stirring the yoghurt by hand

(Fig. 8.3). The Airag in the tank can then be used to make whole-fat milk tofu, Aarchi and milk liquor.

For all yoghurt making, it is necessary for nomadic people to preserve the starter culture over winter for use the following year. They seal 100 g of millet in a small cloth bag and soak this in the yoghurt barrel during fermentation. The millet particles absorb the yoghurt and once they are swollen they can be refrigerated or frozen for preservation (Rose 1989; Wu 1992).

8.2.1.2 Koumiss

Mongolians, Uygurs, Kazaks and other nomadic people in Inner Mongolia, Xinjiang province and other regions of China make koumiss, which means 'fermented mare milk' in Mongolian (Yan and He 2012). It is also referred to as 'Chige' or 'Chigo' by some indigenous people. Koumiss is an alcoholic mare milk beverage fermented by LAB and yeast (Danova et al. 2005). The development of koumiss is a key part of Mongolian dietary customs and their nomadic lifestyle. In the Mongolian diet, semi-liquid food is very important as it easy to process and transport.

Mare milk is different from cow milk. The yield of milk each time is small, but the mare can be milked 5–6 times a day (at 2 h intervals). Generally, one mare

produces 3–5 L of milk per day. A cow yields much more milk on each occasion, but it can only be milked 1–2 times per day. Because of the low yield, nomadic people store the milk in a container and only process the milk when there is sufficient. In the past, the container used for mare milk was made of leather for transportation on the backs of camels. In this way the milk is shaken naturally and, once it became acidic, it could be drunk directly. Actually, this method produces a kind of crude koumiss and the process was likely to have been discovered as a consequence of normal nomadic life.

Koumiss can only be made for about 2–3 months in the summer from July to September because this is the lactation time of mares (Zhang 2007). It is a white or slightly yellow emulsion of uniformly suspended particles with a slightly acidic and full bodied flavour. It can relieve thirst, is tasty and refreshing, and has a high nutritional and medical value. For these reasons, it has been popular with nomadic people for thousands of years and is still popular in Eastern Europe, Mongolia and the provinces of Inner Mongolia and Xinjiang in China (Jin 1998).

The species composition of microorganisms in koumiss is complex (Ni et al. 2007; Wu et al. 2009a; Yu et al. 2011). Multiple factors such as the local environment, climate, production method, fermentation temperature and time all influence the composition of microorganisms and the flavour of the final koumiss product. The microflora in koumiss varies greatly between different regions (Mu et al. 2012). By using different fermentation styles and times, the flavour and microbial flora in koumiss from the same area and same starter cultures might be changed. Koumiss is mainly produced by lactic acid fermentation followed by a short period of alcohol fermentation. The acidity after fermentation is 70–120 °T and the alcohol concentration is around 1–3 % (Zhang 1991; Sun et al. 2003; Li et al. 2006; Chen and Xue 2008).

The detailed production process begins with mare milk and it is essential that this milk is fresh and healthy, preferably freshly milked. The milk is filtered and pasteurised by heating to 90 °C for 30 min. Once the milk has cooled a starter culture (called ‘Horongo’ in the Mongolian language) is added to initiate fermentation. The quality of the final product is directly related to the starter culture which can be a natural starter culture (fresh or from storage) or a mixture of pure LAB and yeast. The quantity of starter culture added depends on the final acidity of the koumiss that is preferred after fermentation. Starter cultures usually include *Lactobacillus bulgaricus*, *Lactococcus lactis* and yeast (Pan et al. 2011; Wu et al. 2009b). The bacteria ferment lactose into lactic acid and carbon dioxide and reduce the pH, making the milk to coagulate and develop an acid flavour. The acidity also prevents invasion of contaminating secondary bacteria that may spoil the flavour and reduce the nutrients (proteins and fat) present. The ratio of *Lactobacillus* species, *Lactococcus* species and yeasts will vary depending on the mare milk used. Nomadic people traditionally make starter cultures from koumiss that they have specially selected for good flavour and quality.

Once the starter culture has been added to the milk, the developing koumiss must be stirred continuously for 20 min at approximately 20 °C until the acidity reaches 68–72 °T. This is achieved in a barrel constructed from wood or leather

Fig. 8.4 Barrel used for koumiss production

(Fig. 8.4) and it is either embedded in soil to a depth of 30 cm or placed indoors to maintain the fermentation temperature around 20 °C. The resulting koumiss is then matured in a cold place (0–5 °C) for 1–1.5 days during which time fermentation continues. After this time the koumiss is ready to drink.

As described previously for yoghurt making, it is necessary for nomadic people to preserve the starter culture over winter for use the following year and they achieve this in the same way: soaking bags of millet in koumiss and then refrigerating or freezing the bag; felt can be used in place of millet bags and then dried. Some people also bury a small piece of koumiss in a jar or store the stirring rod as the starter culture (Zhang 2007). Stored starter cultures must be soaked in pasteurised and skimmed cow or mare milk at 30 °C to be rejuvenated prior to use and then stirred vigorously in a small amount of pasteurised mare milk at 20–22 °C until it is mixed evenly (Fig. 8.4).

8.2.1.3 Kurut

In Chinese elevated areas where cold-adapted grass is found, yaks are the most important livestock animals reared. Daily use of its milk is made in tea and other products. Apart from fresh milk, which is sold to processing factories during the peak lactation period (from June to September), the milk produced in the area is also processed by the indigenous nomads themselves and then stored, consumed or sold. Fermented yak milk (also called 'Kurut') is the most popular dairy product amongst these nomadic people. It is most often produced during the peak lactation period.

When making kurut, the nomadic people always boil the fresh milk first, and then pour it into special covered barrels for natural fermentation. Sometimes they substitute the barrel with a milking barrel with a cover, or a ghee processing barrel. When the temperature of the milk has fallen to 50 °C, they mix it with a volume of kurut from the previous preparation as a starter and stir vigorously (Fig. 8.5). There

Fig. 8.5 Natural fermentation of kurut

are no pure LAB starters in the high, cold grassland and a sample from the previous preparation is always used as a starter. When the temperature of the milk falls to 40 °C the barrel is covered and wrapped with wool to insulate it. During summer and autumn, the fermentation process takes 6–10 h depending on the size of the barrel. Many nomadics choose the best fermentation time based on experience.

There are two kinds of kurut: full-fat kurut and skimmed kurut. The former has the best colour and flavour (Bao et al. 2012a). Kurut is a popular beverage for nomads. It can quench the thirst and provide relief from heat during summer and autumn and has a pleasant and palatable flavour (Zhang 1989).

8.2.2 Processing Methods for Cheese-like Products

Chinese cheese-like products have a solid texture because the whey is drained away from naturally fermented milk products or from milk following acid coagulation. The raw material can be any sort of milk but cow, goat and yak milk are most commonly used. The Mongolians, Daurs, Ewendis and Kazaks living in Inner Mongolia, Xinjiang province and other regions mostly use cow milk and the Tibetans and other ethnic minorities in Qinghai province and the Tibet Autonomous region often use yak milk (Zhang 2007).

8.2.2.1 Milk Tofu

Mongolian milk tofu (known as 'Horod' in the Mongolian language) is a popular traditional food (Zhao and Li 2010). It is made by natural fermentation until the whey and curds have separated and then it is boiled (Zhao et al. 2010a). Milk tofu can be stored for a long time, developing a distinctive flavour that is popular with many different nationalities. Producers can also process the milk tofu by adding starters mainly composed of *S. lactis* and *S. cremoris,* that greatly reduce the fermentation time and increase the quality and stability of the final product.

Fig. 8.6 Traditional milk tofu produced by heating naturally fermented milk

The detailed production procedure for milk tofu begins with raw milk or skimmed milk produced as a byproduct from wurum production and this is pasteurised as described previously. Starter cultures (acidity 0.6–0.7 %) are then added at a rate of 1.5 %, stirred for several minutes and maintained at 30–32 °C until the milk proteins begin to coagulate. To increase the surface area of the coagulated particles and accelerate the removal of whey, the coagulated milk is diluted with further acid starter cultures and maintained until the curds reach the size of beans when the curds are cut. Timing of cutting is crucial for the yield and quality of milk tofu produced. To determine whether the curds have reached the correct size and hardness, a 2 cm by 5 cm long nick is made with a knife. A forefinger is then inserted to a depth of 3 cm into the nick, and, with a stirring action the mixture is tested for regularity and smoothness. If there are no small curds remaining and the whey is clear, it can then be cut. After cutting, the fragile curds are stirred gently with a spoon to ensure they are not broken and then heated. When the acidity of the whey reaches 0.17–0.18 %, the curds will have shrunk to half their original size and the whey can be drained off. The drained curds are placed into moulds for shaping and pressing. There are small vents around the external edge of each mould through which the whey can be further drained. When the moulds are full, weights are applied to the top for ‘pressing’ during which time any remaining whey is removed (Gar 1989; Jin 1987; Zhao and Li 2009).

Most nomadic people use natural fermented set yoghurt as the raw material for milk tofu processing and, once made, it can be eaten immediately, dried as an entire block or cut into small pieces and then dried (Fig. 8.6).

8.2.2.2 Eejig

Naturally fermented set yoghurt is mainly used to produce milk tofu. However, some nomads also use it to make Eejig. In this case, the whey is not drained from the curds. Instead, the coagulating milk is just heated to concentrate the set yoghurt and then dried without shaping. Because of the browning of lactose occurs, Eejig is brown in colour.

Fig. 8.7 Traditional milk cake produced by heating naturally fermented goat milk

8.2.2.3 Aarchi

Aarchi (in the Mongolian language) is a fermented cheese-like product that uses stirred yoghurt as the raw material. It is made by heating the naturally fermented stirred yoghurt to coagulate the protein and produce curds. The curds are then placed in a cloth bag to drain off the whey, shaped and finally dried. If it is shaped into a strip it is called Aarchi but if it is shaped into a block it is called Aarchin Horod. In general, when Aarchin Horod has reached a semi-solid state it is cut into pieces that can either be eaten immediately, or dried (Zhang 2007).

8.2.2.4 Milk Cake

Milk cake is a unique dairy product from Yunnan province that has high nutritional value. Milk cake is a typical fresh cheese-like product made by heating and curdling fresh milk and is rich in casein and lactalbumin, making it increasingly popular (Hu and Huang 2005). Traditional milk cake always uses goat milk as raw material and for natural fermentation (Fig. 8.7). The detailed production begins with filtering fresh goat milk through 4–5 layers of muslin material to remove any foreign material. The milk is heated to boiling point in a pot, and then acid water (either from a previous natural fermentation or another edible acid such as acetic acid) is added and, while still on the heat, the milk is stirred until the protein has fully coagulated. Sometimes, local people use juice from the wild milk vine, *Marsdenia tenacissima* (or the acid water from soaking milk vine, papayas or plums) for curdling (Zhang 2007). The pot is removed from heat when the pH value of whole milk reaches 3.4–3.8 and the curds appear like jellied tofu. Once cooled the whey is drained from the curds. The curds are placed into clean material

Fig. 8.8 Processing procedure for milk fan

beneath a wooden board and pressed for approximately 10 h. When all the water and whey have been drained off, the milk cake is ready to be eaten (Xiao et al. 2007).

8.2.2.5 Milk Fan

Milk fan is a traditional cheese-like product made by the Bai people in Yunnan province. It is milky white or creamy yellow, semi-transparent, smooth, glossy and formed into the shape of a fan (Fig. 8.8). It has a unique flavour. The detailed production method begins with filtering fresh yak milk to which acid water, pre-heated to 50–60 °C, is added. The acid water is the whey from the last production of milk fan that has been placed in an earthenware jar for 3–5 days to encourage natural fermentation and the production of lactic acid. When the pH value reaches 3.45–3.87, it can be used for milk fan production (Xiao and Xu 1996). Once the acid water is added, the mixture is stirred and heated to 63–65 °C until the milk curdles forming coagulated curds. These curds are kneaded, washed and stretched before being placed into a cotton bag and pressed. It can then be dried in the sun. As a variation, some people also use the milk remaining after ghee production to make milk fan.

8.2.2.6 Qula

Qula is a traditional dairy product from the Qingzang plateau (Fig. 8.9). It is made by drying naturally fermented kurut (or the milk remaining after ghee production). Qula is also used as a starter for kurut production in some areas (Duan et al. 2008).

Fig. 8.9 Qula

8.2.3 Processing Methods for Sour Cream Production

There are two procedures used for producing sour cream:

The first is to filter fresh milk into a pot through a clean cloth and then leave it to ferment naturally indoors or in a yurt at 20–25 °C for 1–2 days. A bright yellow creamy layer of butter fat will rise to the surface of the coagulated semi-congealed bean curd-like mixture beneath. The upper layer of sour cream can be removed using a spoon for storage or consumption. The coagulated skimmed yoghurt beneath can be eaten directly or used for making plain milk tofu or low acid dried milk. Alternatively, the skimmed yoghurt can be placed in a yoghurt barrel for further fermentation, or be used for acid milk tofu or dried milk.

The second processing method is to first separate the cream from the skimmed milk using a small milk separator (Fig. 8.10). The separated cream is placed in a cloth bag and the whey or water is squeezed out of it, leaving behind a cream with low water content for eating or making into butter. Adding yoghurt starters into the skimmed milk at an appropriate ratio will then make it ferment and acid coagulate to produce skimmed dry milk or milk tofu (Wu et al. 1996).

8.2.4 Processing Methods for Ghee Production

Ghee can be used in many ways. In addition to being a food, it can also be used as a constituent of medicine and a lubricant. Moreover, it can be used to retain moisture and as a barrier to protect fur clothes. It has been used as a fuel oil inside tents in the frozen grasslands, in lamps and for continuous lighting in lama temples. Women use ghee to protect their skin and artists living in the frozen grasslands mix ghee with various pigments to mould images of Buddha, flowering plants, the landscape, figures, and these have become unique works of art.

To produce ghee, some people use a shaking milk separator, although many others still use a traditional manual procedure in a wooden barrel or leather pouch. In a shaking milk separator, obtaining the butterfat to make ghee is much more

Fig. 8.10 Separating the cream by traditional milk separator

convenient than using traditional methods and it results in ghee with a high fat to water ratio. The quality of ghee produced from this is good, has low water content, is clean and easy to store. The method begins with heating fresh cow milk to 30–35 °C and placing it in the bowl of the milk separator. There are 1–2 layers of cloth in the bowl that filter the milk. The handle is then turned at a steady speed to separate the fresh milk into butterfat and skimmed milk. The butterfat is left to set and, after natural fermentation, it is stirred by hand, removed and placed in fresh water prior to being made into ghee using the traditional methods described below. To increase the ghee yield, some nomadic people add a small amount of yoghurt before heating the milk (Zhang et al. 2013).

Wooden barrel method: Fresh cow milk is placed in a wooden barrel for one day during which time it ferments naturally. On the second day, it is heated to 20 °C and poured into a ghee barrel. There are various sizes of ghee barrel with the largest being 80 cm high with a diameter of 50–60 cm. The diameter at the top is slightly larger than the diameter at the base and there is a cover. In the centre of the cover, there is a small circular hole through which the stirring bar is inserted. Smaller ghee barrels are of the same height (80 cm) but have a smaller diameter (20 cm) and no cover, so the stirring bar can be inserted directly. The stirring bar has a wooden paddle at one end (large paddles have 2–4 holes cut in them) which is placed into the milk. The paddle is gripped tightly by the user and both rotated and moved up and down rapidly to agitate the milk and promote the fat to coagulate and separate from the liquid. The stirring time depends on the amount of milk and the size of the ghee barrel but can be from 1 to 3–4 h. When it becomes difficult to move the stirring bar up and down it is an indication that the butterfat has coagulated into a ball and floated to the top, so stirring can stop. The yellow butterfat floating on the surface is removed and placed in fresh water for rinsing. It is then pressed to remove the water and formed into a rectangular or spherical block of what can then be referred to as ghee. When 50 kg has been accumulated, it is packaged in the skin of a calf or the rumen of an adult yak and stored for at least 1–2 years.

Leather pouch method: This method is similar to the wooden barrel method. Heated milk is poured into a leather pouch made from the skin of a yak, calf or goat. Air is blown into the bag and it is fastened tightly with a string called a lura. The

processor then sits cross-legged on the ground, holds the pouch tightly with both hands, and shakes and rolls it until the butterfat is coagulated into a ball. The pouch is then emptied and the butter fat removed and processed as described before.

8.3 Nutrient Availability and Health Benefits of Traditional Dairy Products

There are many kinds of Chinese traditional fermented dairy products, including yoghurt, cheese-like products, fermented milk curd and ghee. These are made from the milk of cows, mares or yaks and are fermented by LAB, bifidobacteria and/or yeast. During fermentation the components of the milk are degraded, the quantities of soluble phosphorus and calcium are increased and water-soluble vitamins are synthesised. For this reason, their nutritional value is higher than that of raw milk. Moreover, traditionally fermented dairy products have many other beneficial roles, such as moderating the intestinal microflora, increasing the metabolism of protein and vitamins, preventing constipation, producing bacteriocins, reducing the symptoms of lactose intolerance, remedying hepatic injury, reducing the incidence of colon cancer, antitumour activity, enhancing the immune system and reducing the level of cholesterol (Brady et al. 2000; Rolfe and Rial 2000; Vinderola et al. 2002).

8.3.1 Nutritional Value and Health Benefits of Fermented Milk

Fermented milk uses cow milk, goat milk and/or mare milk as the raw material and has a unique flavour (Fig. 8.11). Because of its special flavour and health benefits, fermented milk has become an essential food for nomadic people that cannot be substituted by other products.

8.3.1.1 Nutritional Value and Health Benefits of Yoghurt

Nutritional Components of Milk in General

Milk is the best natural medium for the growth of LAB, it contains all the substances necessary to promote human growth and the nutritional components necessary to sustain human health. Until recently, milk has been considered an ideal food (Luo 1992). The content of protein in milk is 2.8–4.0 %, fat 2.8–4.0 %, lactose 4.6–4.9 %, calcium 0.15 %-0.20 % and phosphorus 0.18–0.26 %. Milk contains all the known vitamins as well (Luo 1992). Moreover, milk is highly digestible, especially the protein components. In milk, the protein digestibility is between 90 and 100 %, whereas, for vegetables and corn it is between 80 and

Fig. 8.11 Kurut, fermented goat milk, koumiss

90 % and in beans 80 % (Luo 1992). So, the nutritional value of milk alone provides a firm basis for the nutritional value of fermented dairy products.

Nutritional Components and Health Benefits of Yoghurt

Yoghurt is the most common dairy product consumed by minority groups in China, such as Mongolians and the Daur nationality (Hu 1990). Through natural fermentation, fresh milk coagulates to become acid curd. For yoghurt under natural fermentation, protein hydrolase produced by LAB can hydrolyse protein into peptides and essential amino acids. Lactates (such as calcium, phosphorus, iron and zinc) that can be absorbed readily during digestion are high in yoghurt and can prevent infant rickets and osteoporosis; specifically calcium, phosphorus and zinc have average values of 1.16, 1.17 and 40.72 mg/kg in yoghurt (Yan et al. 1997). Yoghurt is also rich in Vitamin B, nicotinic acid and folic acid (Guo 2004a). Yoghurt influences the balance of microflora in the human intestinal tract, prevents production of harmful bacteria, reduces the level of cholesterol, improves human immunity, improves the moisture content of the skin, improves eyesight and strengthens teeth (Kong 2004). Moreover, yoghurt contains many biologically active components such as morphine-like peptides, immuno-regulatory peptides, casein phosphopeptide, peptides that reduce blood pressure and antibacterial peptides. These biologically active factors can enhance the immune function of the human body, promote human health, prevent disease and reduce ageing (Dong et al. 2012; Meydani and Ha 2000).

8.3.1.2 Nutritional Value and Health Benefits of Koumiss

Nutritional Components of Mare Milk

Mare milk is rich in nutrition; its total nutritional value is similar to that of human milk (Table 8.1). The proteins in mare milk contain all the essential amino acids necessary to form human protein structures. Mare milk can also be considered as albuminous milk and its digestibility can reach 97 %. Lactose content is up to 6.7 %,

Table 8.1 Comparison of nutritional components of human milk, mare milk and cow milk

Nutritional components (per 100 mL)	Human milk	Mare milk	Cow Milk
Dry material/g	1.24	1.14	1.25
Protein/g	1.06	1.84	3.09
Casein/g	0.33	0.83	2.49
Lactalbumin/g	0.70	0.66	0.70
Aminoethylsulfonic acid/mg	6.30	13.27	0
Lactose/mg	7.10	7.83	4.70
Fat/mg	3.40	2.00	3.80
Calcium/mg	30	9	137
Iron/mg	50	60	45
Zinc/mg	118	700	390
Selenium/mg	0.02	1.71	0.04
Vitamin E/mg	0.26	0.11	0.06
Vitamin B_1/mg	0.01	0.03	0.04
Vitamin B_2/mg	0.04	0.02	0.16
Vitamin C/mg	5.0	30.9	7.0

which is 30 % higher than that of cow milk. There are 20 different fatty acids in mare milk, most of which are highly unsaturated, and are at levels 4–5 times higher than in cow milk. Mare milk can neutralise the adverse effects of saturated fatty acids on the stomach and prevent the metabolism of cholesterol; it can also reduce levels of blood lipid and consequently prevent atherosclerosis. Moreover, the fat in mare milk can be bacteriocidal and prevent the growth of tubercle bacillus. There are 8–14 mg of vitamins in 100 mL of mare milk, which is seven times greater than cow milk, which in turn is greater than any other animal's milk.

Nutritional Components of Koumiss

Koumiss uses mare milk as raw material, mainly fermented with LAB, yeast. Koumiss is a white or bright yellow suspended liquid with no foreign matter or curds. Through the fermentation process, mare milk changes a lot. The lactose content in mare milk is between 6 and 7 % but in koumiss it is between 1.4 and 4.4 %. However, the changes in other substances (such as protein or fat) are not so obvious (Sukhov et al. 1986). LAB present in koumiss degrades lactose into lactic acid and carbon dioxide and this reduction in lactose produces the special koumiss flavour.

Health Benefits of Koumiss

Beneficial medical effects include promoting blood flow, reducing tension, promoting digestion and invigorating the stomach. Drinking koumiss is beneficial for digestion because it prevents growth of harmful bacteria in the intestinal tract, clears

harmful substances from the intestinal tract, prevents and treats various intestinal diseases, reduces the level of blood fat and cholesterol, prevents cardiovascular diseases such as arteriosclerosis, coronary heart disease and hypertension and other diseases such as tuberculosis, scurvy and trace element deficiency. Based on medical experiments, it has been recorded that koumiss can exert obvious adjunctive therapy effects against many diseases, such as hypertension, hyperlipemia, coronary sclerosis, anaemia, pulmonary tuberculosis, chronic alimentary infection, diabetes and neurogenic diseases. A number of Chinese medical studies have recorded that mare milk is sweet, cool, nourishes the blood, moistens the skin, alleviates fever and relieves coughing. Sukhov et al. (1986) researched changes in the intestinal microflora of chronic enteritis patients treated with koumiss and the results showed that koumiss positively influenced the composition of intestinal microflora by reducing the numbers of *Escherichia coli*, *Bacteroides* spp. and *Staphylococcus* spp. in the small intestine and increasing numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. in the large intestine, which also relieved the symptoms of related diseases and cured stomach ache. In this way, koumiss has therapeutic effects on detrimental intestinal bacteria and can substitute for other antibacterial agents. Pan et al. (2011) reported that koumiss reduced the level of blood cholesterol and improved nutritional status by synthesising Vitamin B and promoting the absorption of calcium. Zhangabylov et al. (1985) reported that treating chronic colitis patients with koumiss could normalise their intestinal microflora, promote the absorption of Vitamin B_{12} and increase the amount of Vitamin B_{12} in the blood. Koroleva (1988) reported that koumiss had a specific role in immunity against *Mycobacterium tuberculosis* in horses and prevented infection, so koumiss could not only be considered as an ideal adjunct therapy, but also a naturally occurring drug for tuberculosis. Moreover, koumiss can also be used to treat gastrointestinal diseases, bronchopneumonia and dry pleurisy; if a patient drinks koumiss during the convalescence period, recovery is improved. Fedechko et al. (1995) reported an anti-immunosuppressive action of koumiss showing that koumiss enhanced the antigenic immune response of mice and chickens. Koumiss can also play an anaesthetic role, and because there are exopolysaccharides (EPS) in koumiss, it also has antitumour activity (Yang et al. 1996).

8.3.1.3 Nutritional Components and Health Benefits of Fermented Yak Milk

Yak milk contains 18 amino acids, 8 of which are essential amino acids for human beings. Compared with regular cow milk, the amino acids content is 15 % higher, calcium is also about 15 % higher, vitamins 6 % higher and trace elements such as lactoferrin and lysozyme are high. Yak milk also contains conjugated linoleic acid (CLA) which is considered an anti-atherosclerosis compound. It can also reduce the content of cholesterol, triglyceride and low density lipoprotein in blood,

Fig. 8.12 Traditional cheese-like products

regulate blood sugar, increase bone density, strengthen muscle tissue, reduce fat accumulation, eliminate overactivity of free radicals and enhance immune effects.

Fermented yak milk is made using LAB (Zhang et al. 2008a). Jin et al. (2007) detected 14 main fatty acids in fermented and fresh yak milk; the contents of myristic acid, palmitic acid, oleic acid and stearic acid were more than 90 % of the total fatty acid content. The proportion of total unsaturated fatty acids in fermented yak milk is 31.7 % and the content of CLA is 0.981 ± 0.481 mg/L, which is higher than that of regular cow milk and yoghurt. The proportion of fatty acids in fermented yak milk is similar to that in fresh yak milk.

8.3.2 Nutritional Value and Health Benefits of Cheese-like Products

8.3.2.1 Nutritional Components of Cheese-like Products

Cheese-like products, made by fermentation and concentration, can be considered as having the highest nutritional value of all dairy products because most of the water from milk is removed (Fig. 8.12). The nutritional components of cheese-like products are abundant as the various nutritional components of fresh milk have been concentrated. The protein content of cheese is about 20–35 %. Totally, 100 g cheese-like products can provide 30–40 % of the daily protein requirement of adults. During the maturation of cheese-like products, there are a series of pre-digestion effects on the protein, so its digestibility increases around 96.2–97.5 %. Some cheese-like products can even reach 100 % digestibility compared to only about 91.9 % for whole-fat milk. The availability of essential amino acids in cheese is 89.1 %, milk is only 85.7 %.

Most cheese-like products contain about 30 % fat. Fat can influence the hardness, flavour and odour of cheese. Some people are afraid that eating cheese-like products may increase the level of cholesterol but in fact the cholesterol content of cheese is very low at less than 100 mg/100 g.

Cheese-like products contain various minerals including calcium and phosphorus; 100 g of cheese contains, on average, 800 mg of calcium which is 6–8 times more than that of milk. This provides 30–40 % of the daily calcium requirement and 12–20 % of the phosphorus requirement of adults. Cheese-like products are one of the best foods for use as a calcium supplement. Most cheese-like products are rich in Vitamin A, Vitamin B_2, Vitamin B_{12} and folic acid; 100 g cheese can contain 1,200 IU Vitamin A which is 30–40 % of the daily requirement of adults.

8.3.2.2 Health Benefits of Cheese-like Products

Because cheese-like products are rich in various nutritional components, easy to digest and absorb and not prone to encourage obesity, they are considered as an ideal food. They are sometimes called 'golden milk' and 'pearl of the dairy industry's imperial crown' by nutritionists in China. During the fermentation and maturation process, the original protein in the milk is degraded into essential amino acids and other lower molecular weight substances and fat is decomposed into fatty acids. Among them, 40 % are unsaturated fatty acids, which are not only the structural component of cells, but can also reduce the level of serum cholesterol with associated health benefits such as preventing angiocardiopathy, hypertension and hyperglycemia. Cheese-like products are rich in calcium, with a good ratio of calcium to phosphorus that can be easily absorbed by human beings and benefit the growth of children's bones and teeth, prevent osteoporosis in women and older people and prevent dental caries.

Vitamin A and Vitamin B_2 in cheese can increase immunity and disease resistance, protect the health of the eyes and protect the skin. LAB and their metabolic products produced during fermentation sustain the balance and stability of normal intestinal microflora, promote digestive function and prevent diarrhoea and constipation. Moreover, most of the lactose produced during processing is expelled along with the whey and the remaining is degraded into galactose and glucose. This makes the content of lactose in cheese-like products very low (only 1–3 g/100 g), and so is suitable for consumption by patients with lactose intolerance and diabetes (Guo 2004b).

8.3.2.3 Nutritional Components and Health Benefits of Milk Tofu

Mongolian milk tofu (fermented milk curd) is a traditional national food that can be eaten directly (Fig. 8.13). Milk tofu is rich in protein, mainly casein (about 80 %), and has a balanced ratio of amino acids with digestibility around 100 %. It is reported that the proportion of essential amino acids is 36.1–37.7 % of total amino acids making milk tofu a good protein supplement. Milk tofu is also rich in calcium, phosphorus, iron and zinc, and contains a number of trace elements. These are all easy to absorb in the gastrointestinal tract, making them a useful supplement for elderly people and children.

Fig. 8.13 Mongolian milk tofu

Fig. 8.14 Sour cream

8.3.3 *Nutritional Components and Health Benefits of Sour Cream*

Cream is the fat portion isolated from whole milk. Currently, we can acquire cream with different fat contents by isolating fat and non-fat solids from fresh milk (Fig. 8.14). However, there is still some protein, ash, calcium and phosphorus that remain in the product as well as fat. Sour cream results from the LAB fermentation of cream. The fat content is generally lower than 25 %, although some lightly fermented products may contain more than 40 % fat. There are many kinds of volatile fatty acids in sour cream that may influence its flavour. The unsaturated fatty acids include a number of essential fatty acids, such as linoleic acid, linolenic acid and arachic acid. Linoleic acid is especially important because it is necessary for the synthesis of cephalin. It can also prevent eczema-like dermatitis in infants. When milk is separated into cream and skimmed milk, about 70 % of the phospholipid is found in the cream. So, sour cream is a good source of essential fatty acids and phospholipids and therefore has high nutritional value.

8.3.4 Nutritional Components and Health Benefits of Ghee

Ghee is crude butter made by extracting the upper layer of fats that are bright yellow in colour, following extensive agitation of yak milk. In general, ghee contains 84–87 % butterfat, 12–15 % water and less than 1 % milk protein. Ghee has high energy content and because most Tibetans live in cold regions, ghee has become a very important energy source. The use of ghee in other products is extensive and includes ghee tsampa, ghee tea, ghee cake, sweets and fried vegetables.

8.3.4.1 Nutritional Components of Ghee

Yu et al. (2006) analysed the fatty acid composition of Tibetan ghee using gas chromatography. The results showed that the content of functional fatty acids in ghee, such as CLA, eicosapentaenoic acid, docosahexaenoic acid, α-linolenic acid and arachidonic acid, are high. Comparisons of the fatty acid content of Tibetan ghee and regular cream showed that the content of total CLA in ghee was twice as that of regular cream. This result is similar to the results of Neupaney et al. (2003). Using microwave solution-atomic absorption spectrometry, the contents of calcium, magnesium, iron, copper and zinc in Tibetan ghee was 318, 198.4, 64.9, 7.52 and 20.6 μg/g, respectively (Shi et al. 2007). Ghee also contains cholesterol but regular consumption of ghee is unlikely to cause any increase in serum levels (Kumar et al. 1999). Thirty five volatile compounds have been isolated and identified from Tibetan ghee using a combination of gas chromatography and mass spectrometry (Shi et al. 2006). Of these, the specific flavour of ghee can be attributed to 10 carboxylic acids, 9 esters, 6 alcohols, 2 aldehydes and other trace compounds.

8.3.4.2 Health Benefits of Ghee

Ghee contains a large quantity of butterfat and trace elements; 500 g of ghee can produce 4,000 kcal energy. It is considered as a nutritional and beneficial food necessary for the survival of people who live in extremely cold regions. Ghee contains unsaturated fatty acids such as linoleic acid that can reduce blood pressure (Yue and Wen 1998). Because of the health benefits of CLA, ghee is added to health-care products and functional foods and even used in clinical treatments (Ip et al. 1994; Scimeca and Miller 2000; Truitt et al. 1999). The α-linolenic acid (LNA) present in ghee can produce eicosapentenoic acid (EPA) and docosahexenoic acid (DHA) which are physiologically active in the human body. EPA is widely used to prevent cerebrovascular disease in middle-aged and elderly people and DHA (sometimes referred to as 'brain golden') can provide health benefits such as promoting intelligence and wisdom. It can also affect human growth

(Dai et al. 1998; Tao and Li 2000). Metabolism of arachidonic acid can influence nerve cells (such as adjusting the neuronic transmembrane signal, regulating the release of neurotransmitters and controlling absorption of glucose), and have positive effects on the growth of newborn babies (Boersma et al. 1991), so it is widely added to milk powder and other foods for infants. Icosapent and DHA are essential fatty acids for humans and can prevent thrombosis, reduce blood fat, prevent atherosclerosis, reduce tumours and promote brain growth (Chen 2001; Wu et al. 1997).

According to scriptures, the properties and flavour of ghee are sweet, safe and benefit the lungs, spleen and stomach. The main benefits and uses of ghee are for skin moisturisation, promoting the production of bodily fluids, elimination of wind, relief of fatigue, lung disease, rheumatic arthralgia and pruritus of the skin. Ghee can also lubricate the intestinal tract, promoting peristalsis. Eating half a cup of ghee can relieve an uncomfortable stomach/intestine and allow it to ventilate. A small spoonful of hot ghee can cure disrupted fetal disease and prevent diarrhoea in newborn infants. Because the input amount is small, there are no side effects and it can also be used to treat constipation in the elderly. Research suggests that ghee as a medicine can invigorate the kidneys, promote intelligence and wisdom and is safe. During winter, herdsmen have used ghee to revive people and animals with hypothermia.

8.4 Diversities of Lactic Acid Bacteria

LAB metabolise carbohydrates to produce lactic acid. They include species necessary for the production of fermented dairy products including yoghurt, fermented milk beverage, sour cream and cheese-like products. Moreover, some LAB species are very important probiotics; they have many health benefits, such as improving the intestinal microflora, improving human immunity, anti-tumour activity, reducing blood cholesterol and blood pressure (Wang et al. 2006). This has ensured that they are very important species in the modern food industry. In 1873, Lister first isolated *Streptococcus lactis* from yoghurt, and used this pure LAB strain as the starter for further production (Stark and Sherman 1935). This can be considered as the origin of research and application of LAB as starters. There are abundant LAB sources in China, particularly used by the minority ethnic peoples in nomadic areas (e.g. Inner Mongolia, Xinjiang, Xizang, Chuanxi plateau, Qinghai) who are still making various kinds of dairy products using old, traditional methods; these products include yoghurt, koumiss and kurut (Liu et al. 2011). Traditionally, the beneficial microorganisms in local natural environments were preserved and handed down through the generations. This rich LAB heritage is becoming increasingly valued by more and more dairy scientists and researchers.

8.4.1 LAB Biodiversity in Home-made Koumiss

Koumiss, a fermented dairy product, is a traditional drink of nomadic nationalities. It is a mildly alcoholic, sour-tasting fermented drink usually made from mare milk. It is naturally fermented by a combination of LAB and yeast. It is mainly produced and consumed in Eastern Europe, Central Asia, Southeastern Russia, Mongolia and the provinces of Inner Mongolia and Xinjiang in China. For fermentation of koumiss, LAB and yeast play a very important role and are closely associated with many biological activities and medical health benefits. These beneficial bacteria not only prevent the growth of harmful contaminating bacteria during fermentation, but also produce antibiotic substances that improve the synthesis of Vitamins B_1, B_2 and B_{12} (Jakobsen and Narvhus 1996). LAB biodiversity in koumiss has been widely researched and some potentially probiotic strains have been found that represent a valuable source for the research and future application of LAB.

There are 28 species or subspecies of LAB that have been isolated from home-made koumiss from the genera *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Weissella*. *Lactobacillus* species are the most frequently isolated (Wu et al. 2005, 2009b; Wang et al. 2006; Xu et al. 2006). Twenty two species and subspecies of *Lactobacillus* have been isolated from koumiss samples from different regions. The microflora in home-made koumiss varies depending on the sample region, local environment, the starter used, the processing procedure (fermentation temperature and time) and the season/climate. Moreover, the composition and ratio of different species vary depending on the stage of fermentation even under the same fermentation conditions. Many scientists have studied the biodiversity of the microflora in traditionally fermented koumiss from minority areas in China, and there are many publications. In general, *Lb. helveticus*, *Lb. casei*, *Lb. plantarum*, *Lb. pentosus* and *Lb. kefiranofaciens* are the dominant species in koumiss.

Ishii et al. (1997) isolated 43 strains of LAB form Inner Mongolian koumiss samples. The results showed that *L. rhamnosus* and *L. casei* subsp. *paracasei* can be considered as the dominant microflora. Wu et al. (2009a) isolated 48 strains of LAB from 16 koumiss samples in Inner Mongolia, China. After identification, these lactobacilli strains were identified as *Lb. casei* (17 strains), *Lb. helveticus* (10 strains), *Lb. coryniformis* subsp. *coryniformis* (5 strains), *Lb. paracasei* (3 strains), *Lb. kefiranofaciens* (2 strains), *Lb. curvatus* (1 strain), *Lb. fermentum* (1 strain) and *W. kandleri* (1 strain). Moreover, *Lb. casei*, *Lb. helveticus* and *Lb. plantarum* are the dominant strains. He et al. (2002) sampled 15 home-made koumiss samples from herdsmen's houses, and isolated and identified 20 strains of lactococci, 19 strains of lactobacilli. Menghe et al. (2004) isolated and identified 50 lactobacilli strains from 16 home-made koumiss samples in Inner Mongolia. There were 16 strains of *Lb. casei*, 8 strains of *Lb. plantarum*, 10 strains of *Lb. acidophilus*, 3 strains of *Lb. paracasei* subsp. *paracasei*, 5 strains of *Lb. coryniformis* subsp. *coryniformis*, 1 strain of *Lb. curvatus*, 2 strains of *Lb. kefiranofaciens*, 1 strain of *Lb. fermentum* and 2 strains of unknown *Lactobacillus* sp., *Lb. casei* can be

considered as the dominant species. An et al. (2004) researched on biodiversity of LAB from Inner Mongolia, China, and they found that the dominant strains were *Lb. plantarum* and *Lb. pentosus*. Li and Wu (2002) researched the biodiversity of LAB in home-made koumiss from Xilinguole, Inner Mongolia, China. Among the 12 *Lactobacilli* strains isolated, 6 strains were identified as *Lb. casei*, 3 strains were *Lb. acidophilus*.

Sun et al. (2007) studied the LAB biodiversity of naturally fermented koumiss samples in Xinjiang, China. They isolated 152 strains of *lactobacilli* from 30 samples of naturally fermented koumiss samples, including 78 strains of *Lb. helveticus* (occupied 51.3 %), 28 strains of *Lb. acidophilus* (occupied 18.4 %) and 13 strains of *Lb. casei* subsp. *pseudoplantarum* (occupied 8.6 %). Moreover, they also isolated *Lb. gasseri*, *Lb. casei* subsp. *casei*, *Lb. curvatus*, *Lb. sanfrancisco*, *Lb. coryniformis* subsp. *coryniformis*, *Lb.brevis*, *Lb. plantrum*, *Lb. homohiechill*, *Lb. fermentum*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb.ruminis*, *Lb. crispatus* and *Lb. farciminis*, but in negligible amounts. Sun et al. (2010c) studied the LAB biodiversity of naturally fermented koumiss samples in Xinjiang, China and found that *Lb. helveticus* and *Lb. plantarum* could be considered as the dominant microflora. Hao et al. (2010) studied the LAB biodiversity of koumiss in Xinjiang using DGGE and species-specific primer polymerase chain reaction. Among these koumiss samples, researchers found *Lb. acidophilus*, *Lb. helveticus*, *Lb. fermentum* and *Lb. kefiranofaciens* can be considered as the dominant microflora (Hao et al. 2010). Xiong et al. (2007) isolated 21 strains of LAB from 8 koumiss samples in Xinjiang. Through morphological and physiobiochemical identification, researchers found that 17 strains of them were *Lactobacillus*, including *Lb. casei*, *Lb. alimentarius*, *Lb. jensenii*, *Lb. acetotolerans*, *Lb. pentosus*, *Lb. delbrueckii* subsp. *delbruerkii* and *Lb. zeae*. They also identified 1 strain of *Lactococcus lactis* subsp. *plantarum*, 1 strain of *Enterococcus faecalis*, 1 strain of *Leuconostoc mesentaroides* subsp. *mesentaroides*, and 1 strain of *Streptococcus thermophilous*.

Sun et al. (2010a) also researched on the LAB biodiversity of naturally fermented koumiss from Qinghai. They isolated 11 strains of LAB from 2 koumiss samples, including 7 strains of *Lb. plantarum*, which were the dominant microflora in these two samples.

Table 8.2 shows the composition and distribution of LAB in naturally fermented koumiss from Inner Mongolia, Xinjiang and Qinghai, China.

The above researches showed that the LAB composition in naturally fermented koumiss were highly variable. The composition and content of microorganisms are greatly influenced by the local environment, climate, fermentation temperature, fermentation time and processing procedure. During the fermentation and maturation of koumiss, LAB and yeast composed the dominant microflora (Mu et al. 2012; Zhao et al. 2010b). They can endow the koumiss with special flavour and their metabolites are closely associated with the therapeutic benefits of koumiss.

Table 8.2 Composition and distribution of LAB in koumiss of different regions

Area	Sample No.	Strains	LAB Composition	Amount	Dominant Microflora	References
Inner Mongolia	16	48	*Lb. casei*	17	*Lb. casei*	Wu et al. (2009a)
			Lb. helveticus	10		
			Lb. plantarum	8		
			Lb. coryniformis subsp. *coryniformis*	5		
			Lb. paracasei	3		
			Lb. kefiranofaciens	2		
			Lb. curvatus	1		
			Lb. fermentum	1		
			W. handleri	1		
		117	*Lb. plantarum*	56	*Lb. plantarum*	An et al. (2004)
			Lb. pentosus	39		
			Lc. lactis subsp. *cremoris*	22		
	16	41	*Lb. casei*	14	*Lb. casei*	Sun et al. (2010b)
			Lb. helveticus	12		
			Lb. plantarum	10		
			Lb. diolivorans	2		
			Lb. kefiri	1		
			Lb. reuteri	2		
		43	*Lb. paracasei* subsp. *casei*	35	*Lb. paracasei* subsp. *casei*	Ishii et al. (1997)
			Lb. rhamnosus	4		
			Lb. paracasei subsp. *tolerans*	1		
			Lb. curvatus	3		
	15	53	*Lb. casei*			He et al. (2002)
	16	50	*Lb. casei*	16		Menghe et al. (2004)
			Lb. plantarum	6		
			Lb. acidophilus	10		

(continued)

Table 8.2 (continued)

Area	Sample No.	Strains	LAB Composition	Amount	Dominant Microflora	References
			Lb. paracasei subsp. *paracase*	3		
			Lb. coryniformis subsp. *corniformis*	5		
			Lb. curvatus	1		
			Lb. kefiranofaciens	2		
			Lb. fermentum	1		
			W. kandleri	1		
			W. paramesenteroides	1		
		12	*Lb. casei*	3	*Lb. casei*	Li and Wu (2002)
			Lb. acidophilus	6		
Xinjiang	28	119	*Lb. helveticus*	99	*Lb. helveticus*	Sun et al. (2010c)
			Lb. plantarum	10		
			Lb. casei	4		
			Lb. kefiranofaciens	2		
			Lb. fermentum	1		
			Lb. pontis	1		
	30	152	*Lb. helveticus*	78	*Lb. helveticus*	Sun et al. (2007)
			Lb. acidophilus	28		
			Lb. casei subsp. *pseudoplantarum*	13		
			Lb. casei subsp. *casei*			
			Lb. curvatus			
			Lb. sanfrancisc			
			Lb. coryniformis subsp. *coryniformis*			
			Lb. gasserio			
			Lb. brevis			
			Lb. plantrum			
			Lb. homohiechill			

(continued)

Table 8.2 (continued)

Area	Sample No.	Strains	LAB Composition	Amount	Dominant Microflora	References
			Lb. fermentum			
			Lb. delbrueckii subsp.*bulgaricus*			
			Lb. ruminis			
			Lb.crispatus			
			Lb. farciminis			
			Lb. hilgardii			
	8	21	*Lb. casei*	2	***Lb. pentosus***	Xiong et al. (2007)
			Lb. alimentarius	1		
			Lb. jensenii	2		
			Lb. acetotolerans	1		
			Lb. pentosus	5		
			Lb. delbrueckii	2		
			Lb. plantarum	3		
			Lb. zeae	1		
			E. faecalis	1		
			S. thermophilus	1		
			Lc. plantarum	1		
			Leuc. mesentaroides	1		
Qinghai	2	11	*Lb. plantarum*	7	***Lb. plantarum***	Sun et al. (2010a)
			Lb. helvetics	3		
			Lb. delbrueckii	1		

8.4.2 LAB Biodiversity of Traditionally Fermented Cow Milk and Related Products

Cow milk is a complete food with balanced nutrients. It is considered as the next best food for infants after human and goat milk. Cow milk contains high quality protein, various vitamins and sufficient calcium and lactose. Through LAB fermentation, the content of amino acids and vitamin is increased above the level in the original milk. The content of some minerals (such as iron, potassium, calcium and phosphorus) are also clearly increased by fermentation. Moreover, the beneficial microorganisms present (such as LAB) can inhibit the growth of pathogenic bacteria in the human intestinal tract, and improve the digestion and absorption of nutritive substances. In China, minority ethnic groups use milk to make various kinds of dairy products, including yoghurt, fermented cheese, fermented butter, fermented milk curd and, in Yunnan particularly, a product called milk fan. Most of these dairy products are made by natural fermentation. So, the microbial composition, especially LAB and yeast, play a very important role in the quality and flavour formation of these products.

In China, researchers have studied the biodiversity of LAB in naturally fermented cow milk and related products in the minority areas of China. Zhao and Huo (2008) isolated 71 strains of LAB from 15 samples of traditional yoghurt made by herdsmen's families from Yili and Xinjiang provinces; these included 32 strains of cocci and 39 isolates with rod-shaped cells. The rod-shaped forms were identified as *Lactobacillus* species, and the cocci forms were identified belonging to the genera *Enterococcus*, *Streptococcus*, *Lactococcus* and *Leuconostoc*. *Lactobacillus* species were the dominant microflora in yoghurt from Xinjiang, and *Lb. helveticus* was the commonest species (Zhao 2007).

Liu et al. (2012) isolated 790 strains of LAB from 198 samples of naturally fermented yoghurt (Tarag) from eastern Inner Mongolia, China. Through physiological and biochemical analysis, 16S rRNA gene sequencing analysis and PCR-DGGE analysis, they categorised all the isolates into 31 species and/or subspecies, including 153 isolates of *Lb. helveticus* (19.4 %); 132 isolates of *Lc. lactis* subsp. *lactis* (16.7 %) and 106 isolates of *Lb. casei* (11.0 %). These three species were the dominant LAB species in 'Tarag' in that region.

Airidengcaicike et al. (2010) isolated 171 strains of LAB from 44 samples of naturally fermented milk products in Tibet, China. After systematic analysis, they found these 171 strains could be divided into four genera and 12 species and/or subspecies. The species of LAB from different milk sources were similar. The authors also compared the influence of different geographic settings on the composition of naturally fermented milk products. The results showed that the LAB species in Rikaza region and Naqu regions were different and this was likely because of different climate characteristics (Table 8.3). Mesophilic bacteria such as *Lactococcus* and *Leuconostoc* species can be considered as the predominant microflora in traditional fermented milk products from regions with cold climates (such as Lasa, Naqu), whereas thermophilic bacteria such as *Lactobacillus* and

Table 8.3 Species of LAB and the number of isolates of each species from naturally fermented milk products in Tibet

Species	Area	
	Valley in South Tibet (Rikaza)	Plateau in North Tibet (Lasa, Naqu)
E. faecium	3	1
E. durans	7	9
Lb. fermentum	1	52
Lb. casei	24	25
Lb. plantarum	10	2
Lb. helveticus	2	4
Lb. curvatus	2	0
Lc. lactis subsp. *lactis*	7	4
Lc. lactis subsp. *cremoris*	3	5
Lc. garviea	1	0
Leuc. mesenteroides	0	5
Leuc. pseudomesenteroides	0	0
Total	60	107

S. thermophilus species prevailed in traditional fermented milk products from regions with warm climates (such as Rikaza) (Table 8.3).

Researchers have also studied LAB biodiversity from other fermented cow milk products, such as acid whey from milk fan and milk tofu production. For example, Liu et al. (2009) isolated 91 LAB strains from 20 samples of acid whey collected from Yunnan province, China. They classified these isolates into three genera and nine different species by conventional methods and 16S rRNA sequencing analysis. They concluded that *Lb. helveticus* was the predominant species amongst these samples; mesophilic and thermophilic LAB could be considered as the major microbial components of acid whey in Yunnan province, China (Liu et al. 2009). Ju et al. (2010) isolated 70 strains of *Lactobacillus* species from 15 samples of acid whey in Dali, Yunnan province, China. They found that these 70 isolates could be clustered into four groups: *Lb. fermentum*, *Lb. helveticus*, *Lb. plantarum* and *Lb. caucasicus*. Moreover, *Lb. helveticus* was the predominant species. A further four isolates of *Lb. plantarum* from milk tofu in Inner Mongolia, China have also been screened and found to produce EPS after 48 h in culture (Zhang et al. 2010).

8.4.3 LAB Biodiversity in Kurut and Traditionally Fermented Yak Milk Products

Yak is one of the most ancient and primitive cattle species. It is the only species that can live under the ecological environment of the Tibetan plateau, and is thought to live at a higher altitude than any other mammal. It mainly survives in

the area above 4,000 m altitude in the Tibetan plateau, located in Qinghai, Xizang, Gansu and Sichuan province of China. Small numbers of yak are also found in Mongolia, Russia, Bhutan, Afghanistan and Pakistan. Because of their strong adaptability to the extreme environment on the plateau, yak are also called 'the boat of the plateau'. Because of their limited numbers and narrow distribution, reports on LAB biodiversity of naturally fermented yak milk (which is also called 'kurut') and related products (like qula) is limited. In recent years, research on yak milk products and their microflora composition has increased along with the increasing interest in yak milk products. In China, there are many valuable projects studying LAB and yeast composition of traditionally fermented yak milk in Qinghai, Tibet, Gansu, Sichuan provinces of China.

Zhang et al. (2008b) researched on the chemical and microbial composition of kurut in Qinghai, China. Their results showed that the count of LAB in these samples was 9.18 ± 0.851 lg cfu/mL (colony forming units per mL), and the count of yeast was 8.33 ± 0.624 lg cfu/mL. In subsequent studies in Qinghai, 148 isolates of LAB were collected from 43 kurut samples (Sun et al. 2010a). Moreover, using physiological and biochemical analysis, and 16S rRNA sequencing analysis, the LAB species were identified as *Lb. delbrueckii* subsp. *bulgaricus* (23 isolates), *Lb. helveticus* (13 isolates), *Lb. plantarum* (12 isolates), *Lb. suntoryeus* (1 isolate), *Lb. fermentum* (3 isolates), *S. thermophilus* (51 isolates), *E. durans* (7 isolates), *E. faecalis* (5 isolates), *E. faecium* (1 isolate), *Lc. lactis subsp. cremoris* (5 isolates), *Leuc. lactis* (8 isolates) and *Leuc. mesenteroides* subsp. *mesenteroides* (4 isolates).

Chen et al. (2008) isolated 19 strains of LAB (15 strains of *Lactobacilli*, 4 strains of *Lactococci*) from five kurut samples in Tibet, including *Lb. paracasei*, *Lb. fermentum*, *Lb. brevis*, *Lb. reuteri*, *Lb. delbrueckii* subsp. *bulgaricus* and *P. acidilactici*. Moreover, *Lb. paracasei* was the dominant species. Based on a further 54 samples of fresh yak milk and kurut samples from Tibet, *Lb. fermentum*, *Lb. helveticus* and *Lb. curvatus* were the dominant LAB species (Airidengcaicike et al. 2010).

Bao et al. (2012a, b) systematically analysed LAB biodiversity in various yak milk products from the nomadic residential area of Gannan in Gansu and Hongyuan prairie in Sichuan. They collected 319 isolates of LAB from 88 samples of yak milk products (including kurut, qula, raw milk, whey and butter) from Gansu province (Table 8.4). These isolates were classified into six genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus* and *Weissella*) and 21 species. They considered *Lb. helveticus* (87 isolates), *Leuc. mesenteroides* subsp. *mesenteroides* (49 isolates), *S. thermophilus* (39 isolates), *Lb. casei* (31 isolates) and *Lc. lactis* subsp. *lactis* (19 isolates) to be the predominant species in yak milk products (Bao et al. 2012a).

In the same study, 213 isolates of LAB were collected from 64 samples of traditional yak milk products (including kurut, qula, raw milk, whey and butter) from Sichuan province, China. These isolates were classified into six genera and 17 species and subspecies (Table 8.5). The distribution of isolates by genus was as follows: *Leuconostoc* (40.8 %), *Lactobacillus* (39.0 %), *Streptococcus* (13.2 %), *Lactococcus* (5.6 %), *Enterococcus* (0.94 %) and *Weissella* (0.46 %). Among

Table 8.4 Distribution of LAB species in various traditional yak milk products from Gansu province, China

	Kurut (39)	Qula (23)	Raw milk (15)	Whey (9)	Butter (2)	Total LAB
Rod (164 isolates)						
Lb. brevis	1	1			2	4
Lb. casei	17	5	1	6	2	**31 (9.72 %)**
Lb. coryniformis subsp. *torquens*					2	2
Lb. delbrueckii subsp. *bulgaricus*	11					11
Lb. diolivorans				1		1
Lb. fermentum	6	1	1	1	1	10
Lb. helveticus	**50 (30.12 %)**	**23 (46.0 %)**	4	**10 (27.78 %)**		**87 (27.27 %)**
Lb. hilgardii				1		1
Lb. kefir	4			2		6
Lb. plantarum		2	2			4
Lb. rapi	1					1
Lb. uvarum	4					4
Weissella viridescens			1	1		2
Cocci (155 isolates)						
Lc. lactis *Lc. lactis* subsp. *cremoris*	4			2		6
*Lc. lactis*subsp. *lactis*	9		8	2		19
Lc. raffinolactis	5		8			13
Leuconostoc citreum				1		1
Leuconostoc lactis	6	6	4	2	1	19
Leuconostoc mesenteroides subsp. *mesenteroides*	**21 (12.65 %)**	1	**21 (37.50 %)**	5	1	**49 (15.36 %)**
Streptococcus thermophilus	26 (15.66 %)	7	4	2		39
Enterococcus durans	1	4	1		2	8
Enterococcus faecium			1			1
Total	166 (94 rod, 72 cocci)	50 (32 rod, 18 cocci)	56 (9 rod, 47 cocci)	36 (22 rod, 14 cocci)	11 (7 rod, 4 cocci)	319 (164 rod, 155 cocci)

Table 8.5 Distribution of LAB species in various yak milk products sampled in Sichuan province, China

	Kurut (20)	Raw milk (15)	Qula (18)	Whey (10)	Butter (1)	Total LAB
En. faecium	1		1			2
Lb. buchneri		1				1
Lb. casei	7	1	7	1		16
Lb. delbrueckii subsp. *bulgaricus*	5		4	2		11
Lb. fermentum	1		5	2		8
Lb. helveticus	7	4	22	8		41 (19.1 %)
Lb. plantarum		5				5
Lb. uvarum				1		1
Lc. lactis subsp. *cremoris*		1				1
Lc. lactis subsp. *Lactis*	5	1	1	1		8
Lc. raffinolactis		1	1	1		3
Leu. citreum		2		1		3
Leu. Lactis	5	13	3	2		23
Leu. mesenteroides subsp. *mesenteroides*	4	29	19	8	1	61 (28.5 %)
Strep. thermophilus	20	1	7			28
Weissella cibaria			1			1
Total	55	59	72	27	1	213

these isolates, *Leuc. mesenteroides* subsp. *mesenteroides* (61 isolates, about 28.6 %) and *Lb. helveticus* (41 isolates, about 19.2 %) were the predominant species (Bao et al. 2012b).

The production and consumption of yak milk products are concentrated in Tibet, Qinghai, Gansu and Sichuan provinces of China. Table 8.6 summarises the LAB biodiversity of kurut and fermented yak milk products in these major production areas of China.

8.4.3.1 LAB Biodiversity in Traditionally Fermented Camel's Milk and Related Products

Camel milk has always been considered as a highly nutritious beverage of significant medical value. It has been reported to comprise five kinds of minerals and four kinds of trace elements. On average, 100 g of camel milk contains 116 mg calcium, 12.3 mg magnesium, 87.4 mg phosphorus, 67.7 mg sodium, 14.4 mg potassium, 0.14 mg copper, 0.23 mg iron, 0.08 mg manganese and 0.59 mg zinc. Its composition is similar to that of cow milk, it contains all the essential nutrients for humans. The content of carbohydrates in camel milk is lower than that of cow milk, but the contents of potassium, magnesium, manganese, iron, copper, sodium and zinc are higher. The content of Vc is three times of that in cow milk and the

Table 8.6 The LAB species in naturally fermented yak milk products

LAB species	Xizang	Qinghai	Sichuan	Gansu
E. durans	+	+	–	+
E. faecium	+	–	+	+
Lb. buchneri	–	–	+	–
Lb. brevis	–	–	–	+
Lb. casei	+	–	+	+
Lb. coryniformis subsp. *torquens*	–	–	–	+
Lb. delbrueckii subsp. *bulgaricus*	–	+	+	+
Lb. diolivorans	–	–	–	+
Lb. fermentum	+	+	+	+
Lb. helveticus	+	+	+	+
Lb. hilgardii	–	–	–	+
Lb. kefiri	–	–	–	+
Lb. plantarum	+	+	–	+
Lb. rapi	–	–	–	+
Lb. uvarum	–	–	+	+
Lc. lactis subsp. *cremoris*	+	–	+	+
Lc. lactis subsp. *lactis*	+	+	+	+
Lc. raffinolactis	–	–	+	+
Leuc. citreum	–	–	+	+
Leuc. lactis	+	+	+	+
Leuc. mesenteroides subsp. *mesenteroides*	+	+	+	+
S. thermophilus	–	+	+	+

Note: "–" means there is no species; "+" means there is this species

size of fat globule is smaller. However, the absolute nutritional component can change depending on camel species, season, feeding methods and milking condition.

Fermented camel milk ('Shubat' in Hazakh language and 'Hogormag' in Mongolia language) is a kind of dairy beverage fermented by LAB and yeast. Because of the limited number and narrow distribution of camels, there are only a few reports on microflora biodiversity in camel milk.

In China, most of the data are from research undertaken at the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P.R.China. For example, Menghe et al. isolated 30 strains of LAB from naturally fermented camel milk samples in western Inner Mongolia, China. Through traditional identification techniques, they classified these isolates into four genus and 14 species: *Lb. delbrueckii* subsp. *lactis* (1isolate), *Lb. casei* subsp. *casei* (5 isolates), *Lb. plantarum* (4 isolates), *Lb. casei* subsp. *pseudoplantarum* (1 isolate), *Lb. fermentum* (1 isolate), *Lb. curvafus* (1 isolate), *Lb. farciminis* (1 isolate), *Lb. acidophilus* (1 isolate), *Lc. lactis* subsp. *lactis* (2 isolates), *E. faecalis* (2 isolates), *E. avium* (1 isolate), *E. durans* (2 isolates), *Leuc. mesenteroides* subsp. *dextranium* (2 isolates), *Leuc. mesenteroides* subsp. *mesenteroides* (1 isolate) (Menghe 2001).

Table 8.7 The number of species and isolates of LAB from fermented goat milk and related products in China

LAB species	Inner Mongolia	Yunnan	Qinghai
	Fermented Goat Milk	Milk Cake	Fermented Goat Milk
E. durans	7	3	1
E.saccharominimus		3	
Lb. casei	13		
Lb. plantarum	15		
Lb. helveticuss		2	
Lb. delbrueckii subsp. *bulgaricus*		1	12
Lc.garieae		8	
Lc.lactis subsp. *lactis*		55	
Lc.lactis subsp. *cremoris*		2	
S. thermophilus		1	19

Shuang et al. (2004) isolated 55 strains of LAB and 22 strains of yeast from fermented camel milk in Inner Mongolia, P.R. China. Among these LAB isolates, *E. faecium* was the most common species. They also isolated *Lc. lactis* subsp *cremoris*, *Leuc. lactis*, *Lb. acidophilus* and *Lb. helveticus*.

In summary, we can conclude that LAB in fermented camel milk mainly comprise five genera and 32 species and/or subspecies. The predominant species are *Lb. plantarum*, *Lb. casei*, *E. faecium* and *Lb. sakei*. Because of the variability between sources and processing procedures, microbial biodiversity is different from any other milk.

8.4.4 LAB Biodiversity in Traditionally Fermented Goat Milk and Related Products

Goat milk is known as 'The king of milk' by the International Research Community on Nutrition because its protein content is higher than that of cow milk and contains high levels of minerals and vitamins. However, because of the low milk yield per goat, the related products are limited.

The major goat milk production area in China is Yunnan province. Therefore, the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, P.R. China systematically studied naturally fermented goat milk and related products from this province to determine the biodiversity of LAB (Zhang et al. 2008b; Bao et al. 2011). This research, and the research of other groups, led to an understanding of the composition and number of LAB in fermented goat milk and related products (Table 8.7). We can conclude that *Lb. casei*, *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus* are the most frequently found species but that *Lc.lactis* subsp. *lactis* and *S. thermophilus* are also quite common.

Naturally fermented dairy products occur in a very complicated microecological environment; the microbial composition and dominant species can be specific in a particular product. LAB and yeast are the major microbes in all naturally fermented dairy products, and play a very important role in the production and quality characteristics (He 2010). However, LAB biodiversity and the dominant species vary in the different milk source, geographic setting and processing procedure. Hence, objective and careful research on these microorganisms, especially LAB biodiversity, in these traditionally fermented products can not only provide an important theoretical basis for the quality improvement and industrial production of these naturally fermented dairy products, but can also provide an important biological source for the development of the LAB industry and the dairy industry.

References

Airidengcaicike, Chen X, Du X, Wang W, Zhang J, Sun Z, Liu W, et al. Isolation and identification of cultivable lactic acid bacteria in traditional fermented milk of Tibet in China. Int J Dairy Technol. 2010;63(3):437–44

An Y, Adachi Y, Ogawa Y. Classification of lactic acid bacteria isolated from chigee and mare milk collected in Inner Mongolia. Animal Sci J. 2004;75(3):245–52.

Bao Q, Chen X, Liu H, Zhang W, Liu W, Yu J, et al. Isolation and identification of cultivable lactic acid bacteria from traditional goat milk cake in Yunnan province of China. Afr J Microbiol Res. 2011;5(29):5284–91.

Bao Q, Liu W, Yu J, Wang W, Qing M, Chen X, et al. Isolation and identification of cultivable lactic acid bacteria in traditional yak milk products of Gansu province in China. J Gen Appl Microbiol. 2012a;58(2):95–105.

Bao Q, Yu J, Liu W, Qing M, Wang W, Chen X, et al. Predominant lactic acid bacteria in traditional fermented yak milk products in the Sichuan province of China. Dairy Sci Technol. 2012b;92(3):309–19.

Boersma ER, Offringa PJ, Muskiet F, Chase WM, Simmons IJ. Vitamin E, lipid fractions, and fatty acid composition of colostrum, transitional milk, and mature milk: an international comparative study. Am J Clin Nutr. 1991;53(5):1197–204.

Brady LJ, Gallaher DD, Busta FF. The role of probiotic cultures in the prevention of colon cancer. J Nutr. 2000;130(2S):410S–4S.

Cao YN. The history of Inner Mongolia (2). Hohhot: Inner Mongolia University Press; 2007.

Chen B. Nutrition and food hygiene, vol. 4. Beijing: People's Medical Publisher; 2001.

Chen L, Xue L. The manufacture of Chinese traditional dairy products and its quality control. Beingjing: China Light Industry Press; 2008.

Chen Z, Cheng C, Ma K, Liu G, Li H, LI H. Isolation and identification of lactic acid bacteria from fermented yak milk products in Tibet area. Food Sci. 2008;29:408–12.

Dai C, Yuan S, Liu J. The physiological functions and applications of DHA and EPA. J Microbiol. 1998;18(4):48–50.

Danova S, Petrov K, Pavlov P, Petrova P. Isolation and characterization of *Lactobacillus* strains involved in koumiss fermentation. Int J Dairy Technol. 2005;58(2):100–5.

Dong T, Li H, Yao X, Han Y, Tang X, Che C, et al. Study on the fermentable properties of *Lb. acidophilus* isolated from traditional koumiss of Xinjiang. Xinjiang Agric Sci. 2012;49(8):1531–9.

Duan Y, Tan Z, Wang Y, Cai Y, Huo Y. Characteristics and composing of lactic acid bacteria and microorganisms in yak milk qula. China Dairy Ind. 2008;36(4):27–30.

Fedechko IM, Hrytsko R, Herasun BA. The anti-immunodepressive action of kumiss made from cow's milk. Lik Sprava. 1995;(9–12):104–6.

Gar D. Milk and dairy product technology. Huhhot: Inner Mongolia People's Publisher; 1989.

Guo B. Yogurt. Beijing: Chemical Industry Press; 2004a.

Guo B. Cheese. Beijing: Chemical Industry Press; 2004b.

Hao Y, Zhao L, Zhang H, Zhai Z, Huang Y, Liu X, et al. Identification of the bacterial biodiversity in koumiss by denaturing gradient gel electrophoresis and species-specific polymerase chain reaction. J Dairy Sci. 2010;93(5):1926–33.

Hati S, Mandal S, Prajapati JB. Novel starters for value added fermented dairy products. Curr Res Nutr Food Sci. 2013;1(1):83–91.

He Y. Interaction between lactic acid bacteria and yeasts in traditional fermented milks. China Dairy Ind. 2010;10:43–5.

He Y, Li S, Mu Z, Wang L, Shuang J, Wu J, et al. Isolation, identification, and anti-bacteria function of microorganisms. Trans Chinese Soc Agric Univ. 2002;18(2):91–5.

Hu R. Health protection of fermented milk. Food Process. 1990;2:31.

Hu Y, Huang A. Research on the processing improvement of goat milk cake. Food Ind. 2005;26(4):47–9.

Ip C, Singh M, Thompson HJ, Scimeca JA. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res. 1994;54(5):1212–5.

Ishii S, Kikuchi M, Takao S. Isolation and identification of lactic acid bacteria and yeasts from 'Chigo' in inner Mongolia. China. Animal Sci Technol. 1997;68:325–9.

Jagchid S. New translation and annotation of the secret history of Mongolia. Taipei: Economic Industry Publishing Company; 1979.

Jakobsen M, Narvhus J. Yeasts and their possible beneficial and negative effects on the quality of dairy products. Inter Dairy J. 1996;6(8–9):755–68.

Jin S. Handbook of dairy industry. Beijing: China Light Industry Press; 1987.

Jin S. Informal discussion about milk liquor. China Dairy Ind. 1998;8:34–9.

Jin S, Gong W, Yang M, Zheng Y, Du X, Hong J et al. Analysis of fatty acid composition of homemade yak yogurt. J Southwest Univ Nationalities (Natural Science Edition) 2007;4:794–6.

Ju N, Zhang J, Sun Z, Liu W, Jin Y, Zhang H, et al. The polymorphism of lactobacilli from acid whey for dairy fan in Yunnan area. J Food Sci Biotechnol. 2010;21(5):735–41.

Kong B. Dairy science and technology. Beijing: Science Press; 2004.

Koroleva N. Technology of kefir and kumys. Bull Int Dairy Fed. 1988;227:96.

Kumar MV, Sambaiah K, Lokesh BR. Effect of dietary ghee-the anhydrous milk fat, on blood and liver lipids in rats. J Nutr Biochem. 1999;10(2):96–104.

Li L. Jian wen za ji. Shanghai: Shanghai Ancient Books Publishing House; 1986.

Li S, Wu N. Studies on biological properties of lactic acid bacteria from kumiss in Xilin Guole. J Inn Mongolia Agric Univ. 2002;23(4):27–9.

Li X, Li K, Zou S. Koumiss. Dairy Ind. 2006;7:58–60.

Liu W, Sun Z, Zhang J, Gao W, Wang W, Wu L, et al. Analysis of microbial composition in acid whey for dairy fan making in Yunnan by conventional method and 16S rRNA sequencing. Curr Microbiol. 2009;59(2):199–205.

Liu SN, Han Y, Zhou ZJ. Lactic acid bacteria in traditional fermented Chinese foods. Food Res Int. 2011;44(3):643–51.

Liu W, Bao Q, Qing M, Chen X, Sun T, Li M, et al. Isolation and identification of lactic acid bacteria from Tarag in eastern Inner Mongolia of China by 16S rRNA sequences and DGGE analysis. Microbiol Res. 2012;167(2):110–5.

Luo C. Dairy science and technology. Beijing: Chinese Agricultural Press; 1992.

Matteo R, Nicolas T. Notes of Matteo Ricci in China, vol. 14. Beijing: Chung Hua Book Co; 1983.

Menghe B. Studies on biological properties of lactic acid bacteria from two-humped camel milk and milk products in Inner Mongolia. Hohhot: Dissertation. Inner Mongolia Agricultural University, China; 2001.

Menghe B, Wu R, Wang L, Yang X, Xu J, Dong Y, et al. Isolation and identification of Lactobacillus from koumiss collected in inner Mongolia and People's Republic of Mongolia. China Dairy Ind. 2004;32(11):6–11.

Meydani SN, Ha WK. Immunologic effects of yogurt. Am J Clin Nutr. 2000;71(4):861–72.

Mine Shanren.Yi yu. In: Wang YW,editors. Hei da shi lue and the other four kinds. Shanghai: The Commercial Press; 1937.

Mu Z, Yang X, Yuan H. Detection and identification of wild yeast in Koumiss. Food Microbiol. 2012;31(2):301–8.

Neupaney D, Kim J, Ishioroshi M, Samejima K. Study on some functional and compositional properties of yak butter lipid. Anim Sci J. 2003;74(5):391–7.

Ni H, Bao Q, Sun T, Chen X, Zhang H. Identification and biodiversity of yeasts isolated from Koumiss in Xinjiang of China. Wei Sheng Wu Xue Bao. 2007;47(4):578–82.

Pan DD, Zeng XQ, Yan YT. Characterisation of *Lactobacillus fermentum* SM-7 isolated from koumiss, a potential probiotic bacterium with cholesterol-lowering effects. J Sci Food Agric. 2011;91(3):512–8.

Rolfe, Rial D. The role of probiotic cultures in the control of gastrointestinal health. J Nutr. 2000;130(2S):396S–402S.

Rose A. Fermented foods. Beijing: China light industry press; 1989.

Scimeca JA, Miller GD. Potential health benefits of conjugated linoleic acid. J Am Coll Nutr. 2000;19(4):470S–1S.

Shi Y, Zheng W, Xiong H. Preliminary analysis of aroma components in Ghee from xizang. Food Ferment Ind. 2006;32(2):90–2.

Shi Y, Zheng W, Xiong H. Determination of trace elements of ghee power by microwave digestion-atomic absorption spectrometry. Food Sci. 2007;28(10):440–2.

Shuang Q, Buren T, Miyamoto T. Microflora in traditional fermented camel's milk from Inner Mongolia,China. Milchwissenschaft. 2004;59(11–12):649–52.

Song L. Biography of Wu Zong. The history of the Yuan dynasty. Beijing: Chung Hwa Book Co; 1978.

Stark P, Sherman JM. Concerning the habitat of *Streptococcus lactis*. J Bacteriol. 1935;30(6):639–46.

Sukhov SV, Kalamkarova LI, II' Chenko LA , Zhangabylov AK. Microfloral changes in the small and large intestines of chronic enteritis patients on diet the rapy including sour milk products. Vopr Pitan. 1986;4(7):14–7.

Sun J, He Y, Tian J. Bacteriostatic action of organic acid in the koumiss. J Inn Mongolia Inst Agric Univ. 2003;24:94–7.

Sun T, Wang J, Zhang L, Menghe B, Zhang H. The biodiversity of lactic acid bacteria isolated from koumiss-a traditional fermented mare milk product in Xinjiang of China. Microbiol. 2007;3:451–4.

Sun Z, Liu W, Gao W, Yang M, Zhang J, Wu L, et al. Identification and characterization of the dominant lactic acid bacteria from kurut: the naturally fermented yak milk in Qinghai. China J Gen Appl Microbiol. 2010a;56(1):1–10.

Sun ZH, Liu WJ, Zhang JC, Yu J, Gao W, Jiri M, et al. Identification and characterization of the dominant lactic acid bacteria isolated from traditional fermented milk in Mongolia. Folia Microbiol. 2010b;55(3):270–6.

Sun Z, Liu W, Zhang J, Yu J, Zhang W, Cai C, et al. Identification and characterization of the dominant lactobacilli isolated from koumiss in China. J Gen Appl Microbiol. 2010c;56(3):257–65.

Tao G, Li C. Some summary on α-linolenicacid health effects and application. Food Sci. 2000;21(12):140–3.

Truitt A, McNeill G, Vanderhoek JY. Antiplatelet effects of conjugated linoleic acid isomers. Biochim Biophys Acta. 1999;1438(2):239–46.

Vinderola CG, Mocchiutti P, Reinheimer JA. Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products. J Dairy Sci. 2002;85(4):721–9.

Wang L, He Y, Li S, Wu J, Li S. Study on antibacterial biochemical characterization of *Enterococcus faecalis* in the koumiss. Food Sci Technol. 2006;12:15–8.

Wang RX. The "Hu Shi" waves in the Chinese dietary history. In: Pu MZ,editors. Dietary communication and cultural exchanges. Taipei: Foundation of Chinese dietary culture; 2009.

Wu N. Animal husbandry microbiology. Beijing: China Agriculture Press; 1992.

Wu N, Odongerle, Zhang A, Wang Q, Zhou Y, Tanaka T. The types and technology of national milk products of pastoral areas in Inner Mongolia. J Inn Mongolia Inst Agric Animal Husb. 1996;1:63–7.

Wu H, Zhang G, Cheng Q. Study on composition of fatty acids in soft-shelled turtle oil. J Instrum Anal. 1997;16(2):33–5.

Wu R, Zhang H, Menghe B. 16S rDNA sequence and cluster analysis of *Lb. casei*. Zhang and ZL12-1 isolated from koumsis. China Dairy Ind. 2005;6:4–9.

Wu R, Wang L, Wang J, Li H, Menghe B, Wu J, et al. Isolation and preliminary probiotic selection of lactobacilli from koumiss in Inner Mongolia. J Basic Microbiol. 2009a;49(3):318–26.

Wu R, Wang W, Yu D, Zhang W, Li Y, Sun Z, et al. Proteomics analysis of *Lactobacillus casei* Zhang, a new probiotic bacterium isolated from traditional home-made koumiss in Inner Mongolia of China. Mol Cell Proteomics. 2009b;8(10):2321–38.

Xiao DH. Bei lu feng su. A collection of historical data of Inner Mongolia (3). Hohhot: Local records editorial board; 1985.

Xiao R, Xu K. Processing and refreshing of traditional milk fan in Yunnan. Agric Prod Dev. 1996;6:14–6.

Xiao R, Xu K, Hou Y, Han D, Li H. Experiments of preservation methods for milk cake. Chinese Dairy Ind. 2007;35(9):17–20.

Xie ZZ. Investigations on the five categories of things (Wu za zu). vol 11. Shanghai: Shanghai Bookstore Publishing House; 2001.

Xiong S, Yao X, Tan X, Liu H, Zhang J, Chen X, et al. Biochemical identification of lactic acid bacteria in kumiss. Xinjiang Agric Sci. 2007;44(5):696–701.

Xu J, Yun Y, Zhang W, Shao Y, Menghe B, Zhang H. Fermentation properties of 4 strains of *Lactobacillus casei* isolated from traditionally home-made koumiss in Inner Mongolian of China. China Dairy Ind. 2006;7:23–7.

Yan B, He Y. Symbiotic fermentation characteristics of lactic acid bacteria and yeast isolated from koumiss in Inner Mongolia. Food Sci. 2012;33:131–7.

Yan S, Gaer d, Zhang F, Shuang Q, He Y. Nutritive values of the traditional milk products in Xilingol League of Inner Mongolia. J Inn Mongolia Inst Agric Anim Husb 1997;18(4):31–6.

Yang J, Guo X, Zhang C. Lactic acid bacteria: biological basis and application. Beijing: China Light Industry Press; 1996.

Yao YZ. Miscellaneous notes from the bamboo leaf pavilion, vol. 6. Beijing: Chung Hua Book Co., Inc.; 1982.

Yi LZ. The nationalities of northern China and the origin of the Mongolian nationality (Philosophy and social sciences edition). J. Inn. Mongolia Univ. 1979;34(2):1–23.

Yu DJ. The secret history of Mongolia. Shijiazhuang: Hebei People's Publishing House; 2001.

Yu F, Xing H, Lv P. Fatty acid composition and function evaluation of Tibet yak butter. China Oils Fats. 2006;31(11):35–8.

Yu J, Wang W, Menghe B, Jiri M, Wang HM, Liu W, et al. Diversity of lactic acid bacteria associated with traditional fermented dairy products in Mongolia. J Dairy Sci. 2011;94(7):3229–41.

Yuan J. Research on the imperial banquets of the Yuan dynasty. The study of the history of Mongolia. Hohhot: Inner Mongolia University Press; 2005.

Yue H, Wen J. Determination of fatty acid content in walnat oil. Chem Res Appl. 1998;10(1):79–81.

Zhang R. Chinese yak. Gansu: Gansu Agricultural Press; 1989.

Zhang F. Mare's milk and its processing. China Dairy Ind. 1991;10:226–9.
Zhang HP. Ancient Chinese dairy products. China Dairy Industry. 1994;22(4):161–7.
Zhang D. Tao An meng yi, vol. 73. Beijing: Chung Hua Book Co., Inc.; 2008.
Zhang H. Traditional dairy products for chinese minorities (Review). In:asian indigenous dairy products. Bulletin of the international dairy federation; 2007.
Zhang H, Xu J, Wang J, Sun T, Li H, Guo M. A survey on chemical and microbiological composition of kurut, naturally fermented yak milk from Qinghai in China. Food Control. 2008a;19(6):578–86.
Zhang WY, Yun YY, Sun TS, Menghe B, Zhang HP. Isolation and identification of dominant microorganisms involved in naturally fermented goat milk in Haixi region of Qinghai. China Annal Microbiol. 2008b;58(2):213–7.
Zhang X, Li D, Zhao Y, Niu C, Yang Z. Isolation and screening of exopolysaccharides-producing lactic acid bacteria from Inner Mongolia dairy tofu. Food Sci. 2010;31(1):141–4.
Zhang Q, Xue L, Hu Z. Research progress in traditional ghee. Scie Technol Food Ind. 2013;8:361–4.
Zhangabylov A, Nikolaeva SV, Kalamkarova LI, Il'chenko LA, Muzapbarov B. Effect of dietotherapy incorporating koumiss and shubat on vitamin B12 absorption in the intestines and on its content in the blood of chronic enterocolitis patients. Vopr Pitan. 1985;2:16–8.
Zhao H. Processing and nutrition analysis of wurum and fermented milk curd. J Dairy Sci Technol. 2007;4:175–7.
Zhao R, Huo G. Diversity of lactic acid bacteria isolated in sour milk from Xinjiang. J Shandong Univ (Natural Science). 2008;43(7):1–6.
Zhao H, Li Y. Optimization of fermented milk curd processing technology. J Dairy Sci Technol. 2009;3:133–5.
Zhao H, Li Y. Wurum storage at different temperatures under the conditions of change. J Dairy Sci Technol. 2010;3:122–4.
Zhao H, Gao X, Li Y. Research of influence of eliminate whey temperature on the quality of hurood. J Dairy Sci Technol. 2010a;6:264–7.
Zhao L, Zhang L, Zhang S, Hao Y. Rapid identification of the dominant LAB in fermented koumiss produced in Xinjiang using species-specific PCR. China Dairy Ind. 2010b;38(1):12–5.
Zhuang S. The evolution of Chinese food culture from the eight delicacies. In: Pu MZ,editors. Life and culture. Beijing: Encyclopedia of China Publishing House; 2005.