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Lactic Acid Bacteria in Foodborne Hazards Reduction

Physiology to Practice

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ISBN 978-981-13-1558-9 ISBN 978-981-13-1559-6 (eBook)
https://doi.org/10.1007/978-981-13-1559-6

Library of Congress Control Number: 2018959743

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

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Introduction

Wei Chen and Linlin Wang

Contents

Abstract Food microbiology is the science which studies microorganisms that inhabit, create, or contaminate foods, which normally are of plant and animal origins. Among food-related microorganisms studies so far, probiotic lactic acid bacteria draw extensive attention for their unquestionable importance in food, industry, and health-related fields. This chapter gives a brief introduction to the historical background, habitats, taxonomy, role, and significance of lactic acid bacteria and ends with some thoughts about future development in this field. Taken together, this general introduction hopefully helps the reader to familiarize with the subject and makes the digestion of the more specific aspects easier.

Keywords Lactic acid bacteria · Habitats · Taxonomy · Safety

Food microbiology is the science which studies microorganisms that inhabit, create, or contaminate foods, which normally are of plant and animal origins. Among food-related microorganisms studies so far, probiotic lactic acid bacteria draw extensive attention for their unquestionable importance in food, industry, and health-related fields (Gaspar et al. 2013; Hugenholtz et al. 2002; Lee and Hase 2014; Matthews et al. 2004; Sanders et al. 2013; Turnbaugh 2012). This chapter gives a brief introduction to

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W. Chen, A. Narbad, *Lactic Acid Bacteria in Foodborne Hazards Reduction*,
https://doi.org/10.1007/978-981-13-1559-6_1

the historical background, habitats, taxonomy, role, and significance of lactic acid bacteria and ends with some thoughts about future development in this field.

1.1 Background

The first and most important question is what lactic acid bacteria are. Lactic acid bacteria are normally defined as a group of Gram-positive, catalase-negative, and nonsporulating, aero-tolerant, acid-tolerant, and strictly fermentative cocci or rods bacteria which primarily ferment carbohydrates to lactic acid as one of the main fermentation products (Hugenholtz et al. 2002). Lactic acid bacteria lack cytochromes and are unable to synthesize porphyrins. Its features can vary under certain conditions. Catalase and cytochromes may be formed in the presence of hemes, and lactic acid can be further metabolized, resulting in lower lactic acid concentrations.

Lactic acid bacteria, which are widely distributed in nature, and very rich in biodiversity, are closely related to human production and life. Lactic acid bacteria are valuable biological resources of human and have important social and economic values. Based on reliable archaeological evidence, the history of human use of lactic acid bacteria can be traced back to more than 10,000 years ago. In the long history, the utilization of lactic acid bacteria has made outstanding contributions to the development and practice of human society. For example, thousands of years ago, lactic acid bacteria were widely used in cheese, pickles, yogurt, and other foods and drinks. Among them, yogurt is regarded as "God's gift" by the ancient nomadic people. Although the ancients did not know the principle of yogurt fermentation, they know that yogurt had the magical function of preventing diseases and ensuring health. The scientific research and real application of lactic acid bacteria began in the 1850s. After that, scientists from all over the world made unremitting efforts; therefore, the research on lactic acid bacteria had attracted more and more attention. Russian scientist Metchnikoff who is the Nobel Prize winner put forward clearly in the "longevity theory" that there were a lot of lactic acid bacteria in yogurt. These large number of lactic acid bacteria inhibit the growth of harmful bacteria, reduce the production of bacterial toxins, and play an important role in maintaining health and prolonging life of the residents in the Balkan island. China, which has rich food fermentation resources, is one of the earliest countries to utilize lactic acid bacteria to ferment food. At present, almost every nation has its own lactic acid bacteria fermented food. For example, Mongolian, Kazakh, and Tibetan in Xinjiang, Inner Mongolia, and Tibet have their own traditional fermented koumiss, yogurt sour milk, sour camel milk, and cheese. Milk cake in Yunnan and fermented sour meat and fish (Pradeep et al. 2014) in Hunan, Guizhou, Guangxi, and other regions. Nowadays, people have clearly realized the probiotic function of lactic acid bacteria, and the resources of lactic acid bacteria are being widely applied. Although they are widely present in nature ever in our digestive systems and man had early learned to live together with them for thousands of years, it remains difficult to pinpoint the precise beginnings of human being's awareness of the roles of lactic acid bacteria in our foods. Several significant dates and events in the history of lactic acid bacteria are listed below (Table 1.1).

Table 1.1 Significant events in the history of the lactic acid bacteria

Decade	Event
1000 B.C.E	Start making pickles in China (pickled vegetables)
1780	Scheele identified lactic acid as the principal acid in sour milk (Scheele 1780)
1847	C. Blondeau determined that lactic acid is the product of certain microbial fermentation (Blondeau 1847)
1857	Louis Pasteur showed that microorganisms cause the souring of milk (Pasteur 1857)
1873	Lister isolated *Lactococcus lactis* from sour milk (Lister 1873)
1884	Hueppe first named "yogurt bacteria" as "lactic acid bacteria" (Milth 1884)
1899	Henri Tissier isolated *Bifidobacterium bifidum* from the feces of infants (Tissier 1906)
1900	Ernst Moro discovered *Lactobacillus acidophilus* (Moro 1900)
1905	Stamen Grigoroff isolated *Lactobacillus bulgaricus* from yogurt, that is, *Lactobacillus Bulgaria* (Grigoroff 1905)
1907	Elie Metchnikoff predicted the benefit of LAB in human being (Minot 1908)
1930	Minoru Shirota isolated *Lactobacillus casei*
1935	Minoru Shirota started manufacturing and selling Yakult
1983	Isolated *Lactobacillus rhamnosus* from healthy human
2001	FAO/WHO proposed a definition of "probiotics"
2002	The joint experts of the FAO/WHO in London drafted the guidelines for the evaluation of probiotic in food
2003	People's Republic of China's Ministry of Health No. 84 approved *Lactobacillus reuteri* as a probiotic strain that can be used as a healthy food
2008	The world gastroenterology organization has identified the potential functions of lactic acid bacteria

The prehistorical period is normally divided into the food-gathering period and the food-producing period. Human might remain ignorant of lactic acid bacteria until there was a shift from "hunter-gathering" to "food-producing" agricultural societies in which food storage and preservation became a real big issue. Around one-third of the food supply is lost due to microbe-related spoilage. All food raw materials are contaminated by microorganisms, and the microbial reactions mostly resulted in spoilage of the food, which actually take part in the mineralization of organic materials in nature. To extend the shelf life of foods, the progress of such natural degradation paths must be prevented or delayed, which is mainly by drying or fermenting. Man had early learned to live with microbial-infected food and noticed that lactic acid bacteria might serve as excellent ambassadors for an often maligned microbial world. By serendipity, early humans might find that "spoiled" foods sometimes were still edible or even desired than its original ones. A case in point is food fermentation. Accordingly, food fermentation became an organized activity before 4000 B.C.E. In this connection, lactic acid bacteria are often employed for production of fermented foods including sour milk, yogurt, cheese, fermented sausages, and fermented vegetables such as sauerkraut, pickles, and olives. It is widely accepted that Louis Pasteur was the first person who appreciated and understood the presence and the critical role of food microbes (LAB). He

revealed and isolated the microorganisms (*Lactococcus lactis*) which afforded the souring of milk in 1857 and in 1878. The Nobel laureate Elie Metchnikoff, in one of his books *Prolongation of life*, hypothesized that the longevity of people in the Balkans might due to the bacteria in yogurt in 1907. Minoru Shirota manufactured Yakult by using a special strain of the bacterium *Lactobacillus casei Shirota* in 1935. The term "probiotic bacteria" was proposed in the 1970s and redefined as "Live microorganisms which when administered in adequate amounts confer a health benefit to the host" by the FAO/WHO in 2001. In general speaking, *Lactococcus lactis* is widely accepted as the most important industrial dairy starter microorganism and has been used for hundreds of years. Moreover, interest in probiotic lactic acid bacteria has been rekindled dramatically over the last two decades for their potential health benefits against bacterial infection, diarrhea, IBD, and even tumorigenesis (Wu et al. 2011; Schieber et al. 2015; Sanders et al. 2013; Lee and Hase 2014). In addition, probiotic interventions with disorders (such as diabetes, obesity and metabolic syndrome, nonalcoholic fatty liver disease, etc.) outside the gastrointestinal tract are increasingly recognized (Backhed et al. 2004; Forslund et al. 2015; Llopis et al. 2016).

1.2 The Habitats of Lactic Acid Bacteria

The growth range of lactic acid bacteria is relatively wide. Some species can grow below 15 °C or above 45 °C and also can grow in pH 3~11. This phenomenon shows that lactic acid bacteria have strong adaptability to the environment. Thus it can be seen that lactic acid bacteria are widely distributed in nature, such as in plant materials and their products, different types of fermented foods, fruits, soil, water, cavities (mouth, genital, intestinal, and respiratory tract) of human and animals, and other natural habitats.

Lactic acid bacteria in fermented dairy products mainly include the genera of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*. Generally, the genus *Lactobacillus* is the dominant bacteria. Lactic acid bacteria in sourdough mainly include the genera of *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus sanfranciscensis*, *Lactobacillus crustorum*, *Lactobacillus paralimentarius*, *Lactobacillus mindensis*, *Staphylococcus pentose*, and *Enterococcus faecium*. Lactic acid bacteria in sauerkraut mainly include the genera of *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Lactobacillus*. Lactic acid bacteria in fermented meat products mainly include the genera of *Lactobacillus*, *Enterococcus faecium*, *Leuconostoc mesenteroides*, *Lactobacillus brevis*, and *Streptococcus*.

The genera of *Lactobacillus* and *Enterococcus* are the main lactic acid bacteria in the oral cavity. The genus of *Streptococcus* is the dominant bacteria, leading to the dominant position. The main lactic acid bacteria in the stomach are *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*. Lactic acid bacteria in small intestine mainly include *Streptococcus equinus*, *Streptococcus sanguinis*, *Enterococcus casseliflavus*,

Enterococcus faecalis, *Enterococcus faecium*, *Enterococcus gallinarum*, *Lactobacillus ruminis*, *Pediococcus acidilactici*, and *Bifidobacterium pseudocatenulatum*. Lactic acid bacteria in large intestine mainly include the genera of *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium*. *Lactobacillus* and *Enterococcus* are the main lactic acid bacteria in female genital tract. The genera of *Propionibacterium* and *Streptococcus* on the human skin surface are the predominant bacteria. In addition to digestive tract, urogenital tract, and body surface, there are also a number of lactic acid bacteria in other organs of human. The lung is an important respiratory organ of human. For a long time, the lung is considered sterile. With the development of molecular biology technology and sequencing technology, more and more evidences show that there are a certain number of microbes in the lungs, mainly *Prevotella*, *Sphingomonas*, *Pseudomonas*, *Acinetobacter*, *Fusobacterium*, *Megasphaera*, *Veillonella*, *Staphylococcus*, and *Streptococcus.*

Mice are the most detailed mammalian animals. Through high-throughput sequencing technology, it is shown that there are significant differences in microbial community structure in different segments of the intestinal tract of mice. For example, the Lactobacillaceae is the dominant flora in the stomach and small intestine, and the Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae, and Ruminococcaceae are the dominant flora in the large intestine and feces. *Enterococcus* and *Streptococcus* were initially colonized in the intestinal tract of pigs, and *Lactobacillus* and *Bacteroides* were also colonized in the intestine. *Lactobacillus, Bifidobacterium*, and *Enterococcus* are the dominant bacteria in the gut of pigs, cow, and other animals. Soil and water are also the habitats of lactic acid bacteria, especially in sewage. At present, a variety of lactic acid bacteria are isolated from sewage, including *Lactobacillus ruminis*, *Lactobacillus coryniformis*, *Lactobacillus sharpeae*, *Lactobacillus fermentum*, *Lactobacillus agilis*, *Lactobacillus casei, Bifidobacterium angulatum*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium longum*, *Bifidobacterium breve*, *and Bifidobacterium adolescentis*.

1.3 The Current Taxonomy of Lactic Acid Bacteria

In the last 30 years of the nineteenth century and the first 10 years of the twentieth century, more and more lactic acid bacteria were separated and discovered. At that time, lactic acid bacteria mainly refer to some microorganisms that can acidify milk. Because of the similarity in physiological characteristics and cell morphology, it is very necessary to classify them scientifically and rationally.

It should be pointed out that the term of lactic acid bacteria has no strict taxonomic significance, and thus their members might be heterogeneous from a taxonomic viewpoint. Based on phylogenetic relationship analysis, lactic acid bacteria can be classified into the *Firmicutes* and *Actinobacteria*, including 41 genera as

follows: *Bacillus*, *Halolactibacillus*, *Saccharococcus*, *Brochothrix*, *Listeria*, *Sporolactobacillus*, *Gemella*, *Abiotrophia*, *Aerococcus*, *Alkalibacterium*, *Carnobacterium*, *Desemzia*, *Isobaculum*, *Marinilactibacillus*, *Trichococcus*, *Enterococcus*, *Melissococcus*, *Tetragenococcus*, *Vagococcus*, *Lactobacillus*, *Paralactobacillus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Weissella*, *Lactococcus*, *Lactovum*, *Streptococcus*, *Lachnobacterium*, *Aeriscardovia*, *Alloscardovia*, *Bifidobacterium*, *Metascardovia*, *Parascardovia*, *Scardovia*, *Atopobium*, and *Olsenella.* Other genera that are currently included in the lactic acid bacteria are *Fructobacillus*, *Lacticigenium*, *Pilibacter*, and *Sharpea* which are the new genera of lactic acid bacteria and have not been included in the handbook of *Bergey's Manual of Systematics of Archaea and Bacteria* published in 2015. Notably, *Bifidobacterium* is quite different from other LAB based on 16S ribosomal ribonucleic acid (16S rRNA) sequence-associated phylogenetic relationship analysis (Stefanovic et al. 2017). And the principal genera of lactic acid bacteria are listed below (Table 1.2).

In 1901, Martinus Willem Beijerinck who is the microbiologist and botanist of Holland named *Lactobacillus* as the genera name of lactic acid bacteria (Beijerinck 1901). Then, Orla-Jensen who is a Danish microbiologist published "The main lines of the natural bacterial system" in the *Microbiological Research* magazine in 1909, which is an important progress in the history of bacterial taxonomy (Orla-Jensen 1909). Orla-Jensen wrote a book about lactic acid bacteria in 1919–1943 named *The Lactic Acid Bacteria*. In this book, lactic acid bacteria were systematically classified and described for the first time, which established the foundation of modern lactic acid bacteria taxonomy (Orla-Jensen 1919). It can be said that the publication of Orla-Jensen's book indicated the formation of the science and technology system of lactic acid bacteria and has a great influence on the development of the whole bacterial taxonomy (Orlajensen and Snogkjaer 1940). Based on the idea and method of taxonomy of lactic acid bacteria proposed by Orla-Jensen, Rogosa (Rogosa et al. 1947), Sharpe (Sharpe 1979), Kandler, and Weiss (Barrangou et al. 2011) made a more reasonable and accurate exploration about the classification of lactic acid bacteria according to the characteristic marker enzymes, growth temperature, oxygen demand, environmental physiological preferences, and metabolite types in lactic acid fermentation pathway. In 1971, Lechevalier submitted a classification system based on cytochemical characteristics, which established the foundation of microbial chemical taxonomy. In 1957, PHA Sneath, a British bacteriologist, created a numerical taxonomy method for microbial classification by using the computer

Table 1.2 Principal genera of the lactic acid bacteria

Genus	Cell morphology	Fermentation	Lactate isomer	DNA (mole % GC)
Lactobacillus	Rods	Homo/hetero	DL, D, L	32–53
Lactococcus	Cocci in chains	Homo	L	33–37
Leuconostoc	Cocci	Hetero	D	38–41
Pediococcus	Cocci	Homo	DL	34–42
Streptococcus	Cocci in chains	Homo	L	40
Bifidobacterium	Rods	Hetero		46–67

technology in the classification of bacteria. The relationship between bacterial species was quantified by calculating the similarity coefficient between bacteria. This method enables bacterial classification to go from qualitative description to quantitative analysis (Sneath 1957). In 1969, Mandel reviewed the use of G + C content for bacterial classification. The content of G + C is an indicator of the classification of bacteria. Using nucleic acid hybridization to analyze the homology of DNA, DNA-rRNA molecular hybridization technology is also used for the classification of bacteria, and the application of these techniques and more classification indexes have greatly improved the scientific and accurate classification of bacteria including lactic acid bacteria (Gasser and Mandel 1968; Mandel 1969). Different taxonomic indexes have certain limitations. For this reason, Colwell put forward the concept of polyphasic taxonomy in 1970. Polyphasic taxonomy mainly refers to a taxonomy of microbial classification and phylogenetic evolution by comprehensively utilizing all kinds of different microbial information available. It includes phenotypic information, genotypic information, and phylogenetic information. Polyphasic taxonomy, which covers all the contents of modern microbiological taxonomy, is considered as the most effective means to study the classification of microorganisms and can describe and define all levels of taxonomy.

1.4 The Safety of Lactic Acid Bacteria

Most probiotics are not used safely for a long time. The longest application period is *Lactobacillus acidophilus* and some strains of *Lactobacillus casei*, which have been in the market for 60 years. However, the application time, which many lactic acid bacteria used for producing fermented milk, such as *Lactobacillus delbrueckii, Streptococcus thermophilus*, and *Lactococcus lactis*, is still very short. *Lactobacillus* and *Bifidobacterium*, the most common lactic acid bacteria used in food fermentation for centuries with the aim of increasing the shelf life of foods and improving food safety, do not possess any pathogenic characteristics. Because they are isolated from fermented foods or the human gut microbiota and usually regarded as safe, *Lactobacillus* and *Bifidobacterium* are not involved in the infection process, except for a small number of enterococci. However, there are also rare cases of infection, which are mainly preclinical assessments of new strains or mixed strains, usually as opportunistic infections in people with predisposing conditions (Ouwehand et al. 2003; Lahtinen et al. 2009). For healthy people, there does not seem to be any risk of lactic acid bacteria use; rather, there may be benefits. Therefore, the safety of lactic acid bacteria is very important for high-risk patients and immunodeficiency patients.

It is difficult to identify potential toxic factors of common nonpathogenic microorganisms such as *Lactobacillus* and *Bifidobacterium*. Therefore, potential risk factors have been proposed as an important indicator for evaluating probiotics. Potential risk factors which can partially determine the safety of probiotics are based on the knowledge of potential virulence factors of pathogens. Specific potential toxicity factors are shown in Table 1.3.

Table 1.3 Potential risk factors for some lactic acid bacteria

Relation	Properties	Notes
Metabolism of microbes	Hyaluronidase activity	Important in *Enterococcus*
	Gelatinase activity	
	DNAse activity	
	Mucus degeneration	Mucosal barrier with autoimmune abnormalities
	Formation of D-lactic acid	D-lacto-toxicity
	Amino acid decarboxylase activity	Formation of biogenic amines
Adhesion characteristics	Adhesion to intestinal mucosa	Transfer
	Adhesion of extracellular free proteins	
	The adhesion of essential nutrients and treatment mixtures	
Blood	Resistance to bacteria	Bacteremia
	Erythrocyte dissolution	Anemia
	Hemagglutination reaction	Thrombotic syndrome, endocarditis
Immunology	The composition of cell wall	Arthritis
	Modulate the immune response to inflammation	Inflammation, immunosuppression
The property of microbe	Capsule formation	Resistance to bacteriophore
	Transfer of genetic material	The acquisition of toxic genes

Ouwehand et al. (2003)

The first step in the safety evaluation of lactic acid bacteria is to unequivocally identify the strains correctly. Taxonomy is required to describe a strain, including DNA–DNA hybridization and rRNA sequence determination. In this respect, relevant regulations have been formulated by various countries and organizations. For example, the qualified presumption of safety approach established by the European Food Safety Authority supports the safety of commonly used *Lactobacillus* and *Bifidobacterium*. It provides a basis for genera, species, and strains to be identified as safe. The QPS approach establishes the safety aspects that should be determined and fulfilled for a certain taxonomic unit.

The safety evaluation of probiotics is usually based on pathogen, toxicity, metabolic activity, and intrinsic characteristics of bacteria. Then the safety of the strain was evaluated through in vitro, animal, and human clinical studies. SPF mice or germ-free mice are now used to study the pathogenicity and toxicity of strains.

To ensure the safety of the probiotic strains used, the following experiments should be carried out at least to verify its safety: (1) determine the patterns of antibiotic resistance; (2) assess the metabolic activities (e.g., d-lactate production, bile salt deconjugation) and the side effects during human studies; (3) supervise the post-market epidemiological studies of adverse incidents in consumers; (4) test the toxin, if the strain under evaluation belongs to a species that is a known mammalian toxin producer; and (5) determine the hemolytic activity, if the strain under evaluation belongs to a species with a known hemolytic potential.

In the United States, the Food and Drug Administration (FDA) controls the safety of food and complementary diets. Probiotics, as a regular food and dietary supplement, have been sold in the United States. However, before supplying to consumers, the new microbial strains had to be carefully evaluated for potential health hazards. Lactic acid bacteria have been accepted as safe without any real scientific criteria, partly because they exist as normal commensal microbiota and because of their presence for generations presumably without adverse effect. Currently, harmless probiotics include *L. acidophilus*, *S. thermophilus*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, and *L. fermentum.* They can be used as additives in specific foods such as fermented milk (including yogurt and buttermilk), sour ice cream, and soft cheese.

Most European Union Member States believed that foods that were not considered edible until 1998, such as probiotics, are now considered to be a new type of food. The purpose of the EC new food regulation (258/97) is to ensure the free circulation of new foods and to protect the interests of consumers, especially those who wish to provide information on safety and health. , nutritional value, metabolism, application prospects of new probiotic strains and their prospects, and the level of unqualified substances contained in it is equivalent to the evaluation of the existing ordinary microorganism. These assessments can not be used as criteria for evaluating new foods. However, other aspects of the strain need to be evaluated according to the principles and procedures of the new type of food.

The FAO and WHO convened a joint FAO-WHO Working Group to draft guidelines for evaluating lactic acid bacteria used in food. The working group proposed a framework of strain identification and functional characterization, followed by safety assessment and phase 1, 2, and 3 human trials. It recommended that probiotic foods be properly labeled with the strain designation, minimum numbers of viable bacteria at the end of shelf life, storage conditions, and manufacturer's contact details. The working group further considered that assessment of lack of infectivity by a probiotic strain in immunocompromised animals would increase confidence in the safety of the probiotic.

In summary, the safety testing of lactic acid bacteria mainly includes the following aspects:

1. Lactic acid bacteria strain must be unequivocally identified and defined with correct taxonomy and deposited in a recognized international culture collection for access by manufacturers, scientists, and regulators to ensure organisms can be monitored for genetic drift and comparison with clinical isolates.
2. Novel strains from species with pathogenic, toxigenic, or other adverse properties need to be evaluated with scientific rigor and to be systematically screened for antibiotic resistance and its transference.
3. Immunomodulatory effects of lactic acid bacteria need to be assessed in defined target populations.
4. Clinical studies should comply with the gold standard of randomized, double-blind placebo-controlled design.

5. Lactic acid bacteria in animal feed additives or veterinary products should be evaluated for their safety in the human food chain.
6. Labeling of lactic acid bacteria products should accurately reflect content, shelf life, claimed attributes, and dose.
7. Following the introduction of novel lactic acid bacteria, intake data should be gathered, especially for long-term consumption.
8. Epidemiological surveillance for any associated adverse effects, particularly infection, should be instituted.
9. Characterization of clinical isolates for comparison with endogenous and probiotic strains as integral to confirming its safety.

1.5 The Role and Importance of Lactic Acid Bacteria

Food is an essential part for human life. Food safety is a matter of national economy and people's livelihood. According to the strategy of global food safety established by World Health Organization, chemical harmful substances which include two major categories are the main hazard factors of food safety, such as microbial toxins and heavy metal environmental pollution (Zhai et al. 2014). Microbial toxins can be classified into bacterial toxins and mycotoxins. However, cereals, peanuts, other crops, and animal products are susceptible to mycotoxins, such as aflatoxin, ochratoxin, and other mycotoxins, and foods that have been contaminated by these mycotoxins may induce malignant cancers such as gastric cancer and liver cancer (Duarte et al. 2010; Monbaliu et al. 2010; Delmulle et al. 2005; Ngundi et al. 2005). In addition, microcystins have a strong carcinogenic effect on the liver, and even a very low dose of long-term exposure can cause permanent damage to the liver (Aguete et al. 2001). Toxic heavy metals mainly include lead, cadmium, arsenic, mercury, chromium, zinc, and copper (Yoon et al. 2008). The most toxic to human is lead; it is difficult to discharge from the body, which will cause irreversible loss of children's mental retardation, Alzheimer's disease, and carcinogenesis (Jing et al. 2009).

In developing and developed countries, foodborne diseases which have the most serious impact on children, pregnant women, and the elderly are widely spread and pose a serious threat to health. In addition to its direct health effects, foodborne diseases can cause considerable stress on the health-care system and significantly weaken the capacity for economic production. Diarrhea is the most common symptom of foodborne diseases. Millions of children die from diarrhea every year, and hundreds of millions of children suffer from recurrent diarrhea. Recently, food safety problem has happened frequently in China. The food safety problems do not affect the public health seriously but the development of food industry and even the social stability. Therefore, it is urgent to ensure the safety of food.

In recent years, the function of probiotics has been further developed and analyzed with the in-depth exploration of the physiological characteristics and mechanism of the strains. At present, a considerable number of studies showed that

probiotics have the potential ability to antagonize and reduce the risk factors of food safety. Therefore, we will summarize the research of probiotics in reducing microbial toxins and alleviating the toxicity of heavy metals, so as to provide some reference for the application of probiotics to food safety.

Lead can cause toxicity to multiple organs or systems at the same time. There is a certain relationship between the toxicity and the solubility and form of the compound. Acute lead poisoning can cause abdominal pain, diarrhea, vomiting, headache, dizziness, coma, vasospasm, and liver and kidney damage and can even be life-threatening. Chronic lead poisoning can cause symptoms such as vertigo, anemia, joint pain, and heart failure. So far, there are few articles about lactic acid bacteria to alleviate lead toxicity. However, a large number of studies have shown that some properties of lactic acid bacteria may lead to the ability of lactic acid bacteria to prevent or alleviate the toxicity of lead. The specific research can be summed up in the following four aspects:(1) Lactic acid bacteria can absorb heavy mental ions in vitro, and this ability to absorb heavy mental ions is strain specific. (2) Lead can cause the inactivation of antioxidant enzymes and produce reactive oxygen species (ROS), resulting in oxidative damage to the body. A large number of studies showed that lactic acid bacteria have excellent antioxidant properties and can alleviate oxidative damage caused by various diseases. (3) The bivalent cation such as zinc, calcium, and magnesium can compete with lead and reduce the absorption of lead, while lactic acid bacteria have the function of promoting the absorption of these microelements. (4) Studies have suggested that gut microbes may be the target of toxic of lead. Meanwhile, a large number of animal experiments showed that lactic acid bacteria have the function of regulating intestinal flora, which can also alleviate the lead poisoning by adjusting the intestinal flora.

Cadmium is a kind of heavy metal with strong toxicity, and its incubation period is very long. Acute cadmium poisoning can lead to symptoms such as cough, chest tightness, dyspnea, nausea, vomiting, and abdominal pain. Large doses of cadmium cause acute liver injury and lead to death. Chronic cadmium poisoning involves renal injury (proteinuria, nephrolithiasis, chronic renal failure), bone injury (bone pain, osteoporosis, osteomalacia, spontaneous fracture), reproductive organ injury (testicular and ovarian injury), and cancer (lung, prostate cancer). A large number of studies have shown that lactic acid bacteria may prevent or alleviate the toxicity of cadmium. Its mechanism may be that lactic acid bacteria could combine with cadmium before they are absorbed by the intestine and excreted through feces in order to reduce the absorption of cadmium by human. On the other hand, lactic acid bacteria by enhancing the expression of tight junction, to protect the integrity of the intestinal barrier, maintain intestinal permeability, thereby reducing the absorption of cadmium in intestine.

The existence form of copper is closely related to its toxicity. Under normal conditions, when the intake of copper is 100–150 times greater than that of the human need, it can easily cause toxic reactions and lead to great harm to the human body. The content of copper in liver was the largest, followed by the kidney and brain. When copper enters the brain with blood circulation, it will cause brain damage and affect human's ability to learn and remember. Symptoms of copper poisoning

include a metallic taste in the mouth, accompanied by salivation, nausea, vomiting, hematemesis, and pain in the upper abdomen. Sometimes the excretion is black and the nerve is weak, memory declines, attention is not concentrated, and the temper is irritable and easy to be excited. In addition, copper poisoning can also cause symptoms such as liver swelling or abnormal liver function and induce cancer and even death. Lactic acid bacteria can effectively adsorb heavy metal ions, and it is an excellent antioxidant. It can remove free radicals and alleviate oxidative stress and oxidative damage in the body. Moreover, lactic acid bacteria can be used as a new dietary therapy to treat injury induced by copper. In addition to the three heavy metals mentioned above, the effect of lactic acid bacteria on the reduction of other heavy metals has also been reported, as shown in Table 1.4.

Nitrite is the general name of a type of inorganic compounds. It is usually used as an additive for processing meat products and can inhibit the growth of pathogenic bacteria and spoilage microorganisms. Excessive intake of nitrite will seriously damage human health. At present, the degradation of nitrite is divided into three main categories: physical method, chemical method, and biological method. The mechanism of biodegradation of nitrite is mainly related to the ability of probiotic metabolites to remove nitrite. Probiotics can produce acidic or nitrite reductase during the growth process to degrade nitrite. The mechanism of biodegradation of nitrite is that probiotics can produce acidic substance or nitrite reductase during the growth process to degrade nitrite. It has been proved that *Lactobacillus*, *Leuconostoc mesenteroides*, *Pseudomonas*, *Pediococcus*, *Acinetobacter*, and other probiotics have the ability to degrade nitrite.

Biogenic amine, which is widely used in food, especially fermented food, is the general name of a type of small molecule nitrogenous compounds with biological activity (Cvetković et al. 2015; Guarcello et al. 2015; Li et al. 2014). Excessive intake of biogenic amines can cause damage to the cardiovascular system and nervous system of the human body; it can lead to a rise in blood pressure, faster heartbeat, blood sugar increase, overproduction of adrenaline, and headache (Maintz and Novak 2007). Biogenic amines have good thermal stability, and ordinary cooking

Table 1.4 Lactic acid bacteria with the function of adsorbing other heavy metals

Heavy metals	Lactic acid bacteria	References
Arsenic	*Lactobacillus casein* DSM20011	Halttunen et al. (2007)
Nickel	*Lactobacillus caucasicus* CIDCA8348 and *Lactobacillus caucasicus* JCM5818	Gerbino et al. (2012)
Silver	*Lactobacillus* A09	Lin et al. (2005)
Aluminum	*Lactobacillus plantarum* CCFM 639 and *Lactobacillus rhamnosus* E/N	Yu et al. (2016) and Polakberecka et al. (2014)
Iron	*Lactobacillus plantarum* 299 V, *Lactobacillus delbruecki* Lb-12, and *Streptococcus thermophiles* STM-7	Hoppe et al. (2015) and Sofu et al. (2015)
Zinc	*Lactobacillus delbruecki* Lb-12, *Streptococcus thermophiles* STM-7, and *Lactobacillus*	Sofu et al. (2015) and Mrvčić et al. (2009)

cannot eliminate biogenic amines in food. The main methods for controlling the content of biogenic amines in food are as follows: (1) optimization of production technology and storage conditions, (2) chemical methods, (3) physical methods, and (4) biological technology (Zhang et al. 2015; Fan et al. 2014). At present, the best way to control biogenic amines in food is to inoculate specific microorganisms that can degrade biogenic amines. Bacteria mainly degrade biogenic amines by synthesizing amine oxidase or amine dehydrogenase. For example, *Lactobacillus plantarum* 2142, *Lactobacillus casei* 2763, and *Lactobacillus curvatus* 2771 all have the function of degrading biogenic amines (Rabie et al. 2011).

Nitrosamine is a strong carcinogen, which is widely distributed in many daily consumer goods. Among them, the level of nitrosamine is the highest in tobacco and salted products. It is found that most of the nitrosamines can be degraded by microorganisms. The method of microbial degradation of nitrosamines is simple and inexpensive and will not cause pollution to the products. Lactic acid bacteria can produce some special enzyme which can reduce the content of nitrosamines and nitrite in the fermentation process of pickled products, such as enzyme system that decomposes nitrosamines and nitrite.

Microcystins which have good water solubility and high thermal stability are consumed by drinking water contaminated by microcystins and consuming foods containing microcystins or health products made from blue-green algae. Therefore, microcystins can depend on the food chain to pose a potential threat to human beings. Microcystins have high selectivity and specificity to human liver cells mainly through the blood transfer to the liver, and the liver is the major toxic organ of microcystins. At present, there are three kinds of methods to reduce the pollution of microcystin: physical, chemical, and biological methods. Compared with the physical and chemical reduction methods, the biological reduction method has the advantages of low cost and easy operation. It can be used in algal cells and microcystins at the same time. In other words, it has great potential for the degradation and adsorption of microcystins (Zamyadi et al. 2012; Antoniou et al. 2005). At present, the related research on the eliminating of microcystins by lactic acid bacteria is only in the initial stage. The study found that *Bifidobacterium* (Bb12) and *Lactobacillus rhamnosu*s GG (LGG) have the ability to eliminate microcystins (Meriluoto et al. 2005).

Aflatoxins are secondary metabolites produced by strains of *Aspergillus* parasitized and *Aspergillus flavus* (Zhang et al. 2012). *Aspergillus flavus* and *Aspergillus parasiticus* are common in food, especially in peanuts and corn products. Among the mycotoxins have been found, AFB1 is the most toxic one. Therefore, the research on the mechanism of aflatoxin carcinogenesis is mainly focused on the exploration of the mechanism of AFB1 carcinogenesis. Many studies have shown that many microorganisms have the ability to adsorb or degrade aflatoxins, including lactic acid bacteria, yeasts, and *Bacillus* (Sezer et al. 2013; Oluwafemi et al. 2010). Compared with the traditional physical and chemical methods, biological methods have the advantages of high safety, mild treatment conditions and less damage to the products. Lactic acid bacteria can alleviate aflatoxin mainly through its metabolites to reduce the content and toxicity of aflatoxin, such as bacteriocin and short-chain

fatty acids (Lavermicocca et al. 2003; Ström et al. 2002). On the other hand, lactic acid bacteria can alleviate aflatoxin through inhibition of the growth of Aspergillus flavus and the production of Aspergillus flavus.

Patulin which was first discovered by Glister in 1941 and isolated patulin at University of Oxford were the secondary metabolites produced by fungi (Geiger and Conn 1945). The contamination of fruits, dairy products and feed with patulin is very serious, which poses a great threat to human health. Therefore, many countries have set up a limited standard for patulin in food. Patulin mainly damages the body by reacting with thiol-containing compounds such as glutathione and cysteine. Symptoms of acute poisoning include spasms, convulsions, dyspnea, pulmonary hemorrhage, edema, gastrointestinal ulcers, and congestion, while subacute toxicity is mainly manifested by intestinal dysfunction, including gastrointestinal ulcers, swelling, and bleeding (Appell et al. 2009). At present, researches on the removal of patulin by lactic acid bacteria are at an early stage. In 2007, the Austrian scholar Fuchs first screened out a strain of *Bifidobacterium* VM 12 from 30 strains of lactic acid bacteria, which could better remove patulin in the solution (Fuchs et al. 2008). It was guessed that the adsorption of *Lactobacillus* on patulin may be related to the polysaccharide and protein on the surface of lactic acid bacteria (Hatab et al. 2012).

To explore the effect of lactic acid bacteria in alleviating toxins, we can further develop the probiotic function of lactic acid bacteria and related functional products in order to provide a novel solution for alleviating the toxin effect by dietary strategy.

1.6 Lactic Acid Bacteria, to the Future and Beyond

Life is not sterile, and we live in a microbial world. The microbes were here first and cohabit the planet with us now. Man learned to handle foods in ways that extended their shelf life, and lactic acid fermentation is among the oldest forms of food preservation. Although it sometimes appears that LAB protects our foods, this is by no means their primary role in nature. In the present view of life on this planet, the primary function of all species in nature is self-perpetuation. Therefore, advancements in the application of LAB remains largely lie in exploring their genetics and metabolic studies. LAB fermentations are still used to produce the so-called fermented food today, but preservation is no longer to be the main objective of LAB fermentation. To this end, it is rather the specific taste and texture, maintaining the human health, and promoting benefits that will be the goal of the fermentation in the future (Wu et al. 2011; Turnbaugh 2012; Tian et al. 2015; Cani et al. 2013). Moreover, tomorrow's probiotic LAB might probably move beyond the microorganisms commonly used as probiotic LAB today. For example, LAB is normally nonpathogenic and noninvasive and non-colonizing bacterium. Therefore, recombinant probiotic LAB may represent an interesting direction in the future, especially

to deliver oral vaccine, improve natural immune responses, and restore antigen-specific tolerance. Taken together, dietary supplement of probiotic LAB might have a potential in clinical. Thus, the most exciting era of LAB may lie in its future. Working together, we might create the next epoch in this field. To prepare for this, it is the time for us to gain a foundational understanding of the fields today and learn to love these little creatures that are so small but so smart.

Indeed, fermentation not only improves preservation properties but also confers some special flavors and textures which are quite different from the original ones (Zhao et al. 2011, 2016; Costello and Henschke 2002). In addition, LAB fermentation might also rank as one of the effective biological methods which naturally enhanced food safety such as by destroying naturally occurring toxins, suppressing foodborne illness and allergic reactions, or removing heavy metal ions (Zhai et al. 2013, 2014, 2016; Wang et al. 2011, 2014a, b; Tian et al. 2015; Liu et al. 2013; Guo et al. 2010; Chen et al. 2012a, b; Cheikhyoussef et al. 2008, 2009, 2010). Taken together, LAB is not only of economic significance, flavor preference, and health benefit but is also of value in reducing the toxicity or pathogenicity of foods.

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Chapter 2
Genomic Analysis of Lactic Acid Bacteria and Their Applications

Wei Chen and Zhennan Gu

Contents

Abstract Lactic acid bacteria (LABs) comprise a group of Gram-positive, rod- or cocci-shaped, and low G + C content bacteria with common metabolic and physiological characteristics. The association of LAB with food fermentation can be traced back to the early nineteenth century. As the "milk-souring organisms," they produce lactic acid as one of their main metabolic end products (Orla-Jensen, The lactic acid bacteria, (D Kgl danske vidensk Selsk Skrifter Naturv og mathematisk Afd, 8 Række, vol 2). A. F. Høst, København, 1919). The classical phenotype-based identification of LAB is not always reliable because the phenotype could be subject to the environmental variations. As a more reliable identification method, nucleic acid probe was applied for genotypic tests (Salama M, Sandine W, Giovannoni S, Appl Environ Microbiol 57(5):1313–1318, 1991). The 16 s or 23 s rRNA probes with specific sequences on a phylogenetic basis were both practical and reliable approach to identify LAB in the 1990s (Schleifer KH, Ehrmann M, Beimfohr C, Brockmann

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E, Ludwig W, Amann R, Int Dairy J 5(8):1081–1094. https://doi.org/10.1016/0958-6946(95)00047-X, 1995). With the advancement of sequencing technology and sequence database, LAB genome analyses using sequencing technologies and bioinformatics have not only revolutionized the characterization of lactic acid bacteria but also had a huge impact on interpreting its functional and ecological diversity. This chapter will focus on new development of DNA sequencing, gene-based technologies, and its implication on LAB for the scientific and industry fields.

Keywords Lactic acid bacteria · Sequencing · Plasmid · 16S RNA

2.1 Introduction

Lactic acid bacteria (LABs) comprise a group of Gram-positive, rod- or cocci-shaped, and low G + C content bacteria with common metabolic and physiological characteristics. The association of LAB with food fermentation can be traced back to the early nineteenth century. As the "milk-souring organisms," they produce lactic acid as one of their main metabolic end products (Orla-Jensen 1919). The classical phenotype-based identification of LAB is not always reliable because the phenotype could be subject to the environmental variations. As a more reliable identification method, nucleic acid probe was applied for genotypic tests (Salama et al. 1991). The 16 s or 23 s rRNA probes with specific sequences on a phylogenetic basis were both practical and reliable approach to identify LAB in the 1990s (Schleifer et al. 1995). With the advancement of sequencing technology and sequence database, LAB genome analyses using sequencing technologies and bioinformatics have not only revolutionized the characterization of lactic acid bacteria but also had a huge impact on interpreting its functional and ecological diversity. This chapter will focus on new development of DNA sequencing, gene-based technologies, and its implication on LAB for the scientific and industry fields.

2.2 DNA Sequencing and Its Application on Genomic Analyses of Lactic Acid Bacteria

Forty years ago, a DNA sequencing method based on dideoxy chain termination was established with polyacrylamide gel electrophoresis and fluorescent labeling on a slab gel (Sanger et al. 1977). It was the beginning of a sequence era. Based on this method and its modification, a Human Genome Project (HGP) was accomplished (Lander et al. 2001; International Human Genome Sequencing 2004). The high cost and manpower for the HGP were unsustainable for large whole genome project on a routing basis. Tremendous effort had been made to reduce the manpower and cost of sequencing and leaded to the introduction of so-called next-generation sequencing (NGS).

The coming of NGS technologies has quickly extended DNA sequencing applications from genome-oriented project to many fields, including basic, clinical, and applied research. The newer sequencing technologies combine a number of tactics and strategies that cover from template preparation, sequencing, and imaging to alignment and assembly methods. The merit of NGS is so evident since it can produce a huge amount of sequencing data in a short period of time with a reasonable cost, in some cases over one billion short reads per run (Metzker 2010).

NGS platforms on the market can be classified into two main categories, depending on their reading capacity, short- and long-read sequencing. The short-read technologies (second-generation sequencing) mainly supported by Illumina and Ion Torrent platforms have relative short reading lengths, less than 300 bases per read. Principally, DNA and primers are first attached on a flow cell surface and amplified with polymerase in situ to form the so-called DNA clusters. A single fluorescent-labeled nucleotide is added to the nucleic acid chain in the cluster, and non-incorporated nucleotides are cleaned away. The camera records the fluorescent image of the attached nucleotide. Then the fluorescent dye is chemically removed from the DNA and ready to start the next cycle (Diaz-Sanchez et al. 2013). Illumina supports bidirectional paired-end sequencing to compensate for its short read, which also improves alignment rates compared to one-directional single read (Shawn and Richard 2016). The long-read technologies (third-generation sequencing) are mainly developed by Pacific Biosciences and Oxford Nanopore, with the technology of single-molecule real-time sequencing (SMRT). The SMRT technique is performed on a chip that contains lots of zero-mode waveguide (ZMW). Each DNA polymerase is attached to the bottom of a ZMW with a single-stranded DNA molecule as a template. The signal from incorporated nucleotide labeled with fluorescent dye is detected in real time. When the nucleotide is attached to the DNA, the fluorescent dye is cleaved off and is ready for the next-round reaction. The SMRT sequencing can yield an average read length of 12 kilobases and millions of reads per run (Soorni et al. 2017). Fourth-generation in situ sequencing platforms apply second-generation sequencing technology to read nucleic acid sequence in fixed tissues or single cells directly. The advantage of this technology is that it provides single-cell resolution and is useful for certain applications (Ke et al. 2016). Fast development of DNA sequencing technology has not only reduced cost per project and increased reading efficiency including throughput and accuracy but has also made it being used in almost every aspect of biology and life-related science.

Since the first complete genome sequencing of *Lactococcus lactis* ssp. lactis IL1403 in the early 2000s, more than 100 genome sequences from different species and strains of LAB are available in public databases (Douillard and de Vos 2014; Bolotin et al. 2001). *L. lactis* is used in dairy fermentation as starters for industrial cheese production and is one of the well-known and commonly used microorganisms for studies of LAB physiology. Two main steps were used to sequence the strain *L. lactis* IL1403, sequencing random segments of the genome and applying multiplex long accurate PCR, and generated the sequence of the entire genome with 2.35 Mb size. The project identified 1495 potential protein-encoding genes on the genome map and functionally associated half of these coding genes to known proteins on the basis of homology (Bolotin et al. 1999). As the first genome to be com-

pletely sequenced in LAB, the information from this project helped to reveal and interpret many unknown mechanisms in *L. lactis*, such as origin and terminus of DNA replication, prophages, gene regulations and RNAs, metabolism, transporters, and protein secretion (Bolotin et al. 2001).

Although several genome sequencing projects of LAB had been completed or initiated before 2005, the major progress was made after the introduction of second-generation sequencing or NGS. Many strategies were applied for the completion of genome sequencing with individual LAB, including sequencing with one platform or with a combination of several platforms. For the project of genome sequencing of *Lactobacillus plantarum* ST-III, Wang and colleagues applied combined sequencing approach to generate about 186 k paired-end reads (28-fold coverage of the genome) and 11 Mb reads (262-fold) by Roche 454 and Solexa paired-end sequencer, respectively. They used ABI 3730 capillary sequencer to fill the remaining gaps between the scaffolds. ST-III genome consists a 3.24 Mb circular chromosome and a 53.5 Kb plasmid (Wang et al. 2011). In order to completely sequence the genome of *Lactobacillus plantarum* ZS2058, a strain producing conjugated linoleic acid (CLA), Yang and colleagues used both Roche 454 pyrosequencing and Illumina paired-end sequencing platforms (Bentley et al. 2008; Margulies et al. 2005); produced a total of 199,006 reads (74 Mb) and 4,379,450 reads (442 Mb), respectively; and reached a depth of 23.16-fold and 38.34-fold genome coverage, respectively. The whole genome sequence of *L. plantarum* ZS2058 includes a chromosome with a size of 3.20 Mb and three plasmids (ZS2058p1, 56.9 Kb; ZS2058p2, 11.2 Kb; ZS2058p3, 8.7 Kb). ZS2058 chromosome and its plasmids encode 3050 and 85 potential protein-coding sequences, respectively (Yang et al. 2015). By similar approach, a genome sequence of ND02, a *Lactobacillus delbrueckii* subsp. *bulgaricus* strain, was completed by both 454 and Solexa sequencing platforms. ND02 comprises a circular 2.1 Mb chromosome and a 6.2 Kb plasmid with 2177 and 6 coding genes, respectively (Sun et al. 2011). Because of their speed and capacity, NGS platforms are growingly being used to complete a genome sequencing project or to address certain research interest. In an analysis of comparative genomic of the genus *Enterococcus*, a total of 29 strains of *Enterococcus* species were genome-wide sequenced by Illumina MiSeq (Zhong et al. 2017). Combining these genomic sequences with other eight genomic sequences from the GenBank databases, a genomic comparison of the genus *Enterococcus* was performed. The genome sizes of these strains were varied from 2.3 Mb to 5.3 Mb, with 2154 to 5107 predicted coding genes. The core- and pan-genome from *Enterococcus* genus were defined by using comparative genomic analysis. While the core-genome of *Enterococcus* from 37 strains consists of 605 gene families, the pan-genome of *Enterococcus* extends to 29,545 gene families. Thirty-seven genomes from 34 species were found to form the representative core-genome of *Enterococcus*, and the result of the comparative genomic analysis of *Enterococcus* was consistent with the results from studies on comparative genomics of *Streptococcus* and *Bifidobacterium* (Gao et al. 2014; Donati et al. 2010; Hu et al. 2015). These 605 core-genome sequences code for genes covering various functions, such as animo acid and carbohydrate metablism, cell replication and cell cycle, DNA metabolism, and RNA

metabolism (Zhong et al. 2017). Different with the pan-genome, the numbers of core-genome are relatively stable, and the changes in genome number had little impact on the core-genome number. A similar technical approach with Illumina HiSeq 2000 was also applied to the genus *Bifidobacterium* for comparative genomic analysis. This study compared the genomic diversity of the 45 type strains. The genome sizes of *Bifidobacterium* varied from 1.7 Mb to 3.3 Mb, with predicted coding gene numbers from 1369 to 2564. The pan-gene for all 45 genomes contained larger than 20,000 gene families, and the core-gene families only had 402 gene families (Sun et al. 2015). By comparing the maximum likelihood tree (MLtree) of bacteria using 45 *Bifidobacterium* genomic information with other 426 genera from phyla of bacteria, Sun and colleagues suggested that bifidobacterial species are a family of distinct lineage in the phylum *Actinobacteria* and were inherited from the same most recent common ancestor (MRCA). They also found that the group of *B. asteroides* is the most ancient lineage of bifidobacteria, not the deepest branch of bifidobacteria as previously suggested based on 16S rRNA sequence analysis (Sun et al. 2015; Ventura et al. 2006).

The most recent LAB sequencing project was to study the comparative and functional genomics of the *Lactococcus lactis* using the single-molecule real-time sequencing (SMRT), a sequencing technology established by Pacific Biosciences (Gupta 2008; McCarthy 2010). The SMRT sequencing has made it fast to sequence a large number of bacterial genomes with high quality. Sixteen lactococcal genomes were completely sequenced in this project, twice more than the existing number of fully sequenced lactococcal genomes which is publicly available. The 30 *L. lactis* strains, including 14 obtained from the National Center for Biotechnology Information (NCBI) database, were from different ecological niches: dairy, plant, meat, fermented food, and sink drain isolates. The genome sizes of these strains varied from 2.3 Mb to 2.6 Mb, with 1947 to 2643 coding genes per genome. By comparing the genomic sequences from six different ecological origins, the authors suggested that niche adaptation played an important role of governing the genetic code of each strain (Kelleher et al. 2017).

2.3 Plasmids of LAB and Their Functions

Plasmids are small, double-stranded, circular DNA molecules in cells, frequently found in bacteria. They are independent on the cell chromosome and self-replicative. Functionally, plasmids are not genetic materials for the life cycle of cells but often provide certain functions to assist cells for surviving in a harsh environment or to compete with other cells for the growth advantage (Wegrzyn and Wegrzyn 2002). Self-replicative plasmids could become integrative when plasmids are introduced into a new host cell which provided with a replicon to allow their replication or integrated into chromosome of the host cell. Thereby, plasmids are useful tools for molecular manipulation to express, multiply, substitute, insert, or silence a particular gene of interest (Landete 2017).

2.3.1 New Developments in the Area of LAB Plasmids

The first *Lactobacillus* plasmid was isolated from *Lactobacillus casei* in 1976 (Chassy et al. 1976). Since then, more and more plasmids from different LAB species were discovered and characterized (Gasson 1990). Eleven genera of LAB have been reported to contain plasmids, including *Lactobacillus*, *Bifidobacterium*, *Brevibacterium*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Oenococcus*, *Tetragenococcus*, and *Weissella*. They extremely varied from copy number to size, the copy numbers range from 1 to more than 100 per cell, and the size ranges from 0.87 kb to 250 Kb (Cui et al. 2015; Ainsworth et al. 2014; Mills et al. 2006). Certainly, progress in DNA sequencing will have more LAB plasmids being discovered on gene level and characterized on function level.

Like many other bacterial plasmids, some LAB plasmids carry antibiotic resistance genes. To access the occurrence of tetracycline resistance (TetR) in *Lactococcus lactis* isolated from Polish raw milk, 500 isolates were screened by culture on GM17 plates supplemented with tetracycline. Two strains contain single TetR plasmid, while each of the other four strains contains more than one TetR plasmid (Zycka-Krzesinska et al. 2015). In another study of identification of tetracycline- and erythromycin-resistant Gram-positive cocci derived from dairy food product, 107 coccal colonies from *Lactococcus lactis*, *Enterococcus faecalis*, and *Streptococcus bovis* genera were tested for their resistant capacity to tetracycline judged by minimum inhibitory concentration (MIC) value. Seventeen *L. lactis* showed resistance to both tetracycline and erythromycin judged by their high MIC values. Southern blot analysis showed the existence of plasmid carrying TetR genes in *L. lactis* (Devirgiliis et al. 2010). Acquired antibiotic-resistant genes of lactobacilli have been reported by Klare et al. Among 473 isolates of LAB including the genera *Lactobacillus*, *Pediococcus*, and *Lactococcus*, 17 isolates of *Lactobacillus* were found to be resistant to one or more of tested antibiotics (Klare et al. 2007). Horizontal transfers of antibiotic genes by plasmids or transposons from lactobacilli to other bacteria were also reported. In vitro and in vivo experiments had demonstrated the transfer of *Lactobacillus plantarum* resistance plasmid to *Enterococcus faecalis* and gut microbiota (Feld et al. 2008). Antibiotics are regularly used for stimulating animal growth and preventing plant disease (Ammor et al. 2007; Wegener 2003). Such widely use of antibiotics has a huge impact on the bacterial environment and favors the selection for new antibiotic-resistant strains, including LAB (Bronzwaer et al. 2002; Zycka-Krzesinska et al. 2015). These studies raise the importance for the regular screening of plasmid carrying antibiotic genes in dietary LAB to prevent potential horizontal transfer from LAB to human bacteria.

One major factor to select a good dairy starter strain is its ability to deter phage infection, a major cause for unproductive fermentations (Labrie et al. 2010). Particularly, bacteriophages are resistant to pasteurization and other sanitation measures. Plasmids in LAB provide some protection against bacteriophage attack. Several cellular defense mechanisms are applied to interrupt the life cycle of phage at different stages, including adsorption inhibition (Ads), injection blocking, and restriction/modification (R/M) and abortive infection (Abi) (Mills et al. 2006). Ads

is associated with blocking phage receptors by protein or carbohydrates coded by plasmid genes and is the most common one among all mechanisms (Mills et al. 2006). Numerous plasmids have been identified to have inhibitory effects on bacteriophage adsorption. Although plasmid pME0030 from a phage-resistant *Streptococcus lactis* strain was one of the first being reported that it was able to prevent phage adsorption, its mechanism is still not yet clear so far (Sanders and Klaenhammer 1983). Analysis of plasmid pCI528 which was isolated from *L. lactis* ssp. *cremoris* UC503 found that pCI528 could increase the levels of both rhamnose and galactose in its host cells and exhibited adsorption inhibition phenotype by modifying the cell surface structure of strains harboring the plasmid pCI528 (Lucey et al. 1992). Quiberoni et al. further demonstrated that phage Ads could be achieved by direct incubation of culture with rhamnose, galactose, or other saccharides (Quiberoni et al. 2000). Akcelik and Tunail reported that plasmid p2520L isolated from *L. lactis* ssp. *lactis* P25 was able to block the adsorption of phage, possibly by producing a 30 KDa cell surface protein (Akcelik and Tunail 1992).

Abortive infection (Abi) can be considered as restricted or inhibited phage proliferation after DNA injection. A variety of defense mechanisms are involved to interrupt the life cycle of phage, at the level of replication, transcription, translation, and particle assembly. As suggested by Mills et al., more than 21 plasmid-coded and Abi-related genes have been documented in *Lactococcus* (Mills et al. 2006). Abi mechanism was first reported and designated as AbiA by Klaenhammer and colleagues. They found that plasmid pTR2030 from *Streptococcus lactis* ME2 could induce an abortive phage infection (Klaenhammer and Sanozky 1985). Although the mechanisms of Abi system are not yet fully understood, Abi exerting its action at different stages of the intracellular phage life cycle was reported and characterized, including interfere with replication of phage DNA (AbiA), interfere or delay the expression of phage gene (AbiG or AbiU), delay the gene expression to inhibit the synthesis of phage capsid protein (ABiC), interrupt with protein translation (AbiDi), trigger the accumulation of replicative form of phage DNA (AbiQ), and disturb the protein factors needed for phage replication (AbiS) (Mills et al. 2006).

Injection blocking and restriction/modification is another mechanism used to inhibit phage proliferation. When studying the interaction between an insensitive strain of *Lactobacillus casei* and phage PL-1, Watanabe and colleagues found that there was no bacterial lysis when cells were incubated with phages. Further electron microscopy analysis revealed that phage DNA was still in the capsid on the cell surface, while many empty capsids were found on sensitive strain. Evidently, after the adsorption of phages to the cell surface, phage DNA injection was failed (Watanabe et al. 1984). The DNA injection-blocking phenomenon later was classified as Sie (superinfection exclusion) or Sie-like systems. Garvey et al. were the first to report that plasmid pNP40 from lactococcal could block the injection of Φc2 phage DNA. Pip, a membrane protein, is necessary for c2 phage adsorption to its host *Lactococcus lactis*. It was hypothesized that protein encoded by pNP40 plasmid might interfere the membrane insertion activity of Pip which is necessary for c2 adsorption to the cell surface of Lactococcus lactis and prevent phage DNA injection (Garvey et al. 1996).

After effective injection of DNA, phage infection still could be jeopardized by restriction/modification (R/M) systems. R/M systems mainly have two enzymatic activities, restriction activity (endonuclease) and modification activity (methyltransferase), and four types, according to the molecular structure, cofactor, sequence consensus, and cleavage site. Although sequence analyses suggested that R/M genes are encoded by both chromosome and plasmid DNAs, many R/M systems were related with plasmids in lactic acid bacteria. Three types of R/M systems were reported in lactococci, including types I, II, and III as reviewed in details by Forde et al. (Forde and Fitzgerald 1999).

2.3.2 The Function of LAB Plasmids and Their Relevance to Research

Plasmids are essential tools for molecular biology laboratories where they are used to knockout, insert, and modify target genes to regulate their expressions. Food-grade plasmid vectors are constructed with DNA material from GRAS bacteria to meet the safety standards required by the US Food and Drug Administration (FDA) and the European Food Safety Authority.

LAB food-grade cloning vectors are basically based on replicative and integrative systems (Landete 2017). A replicative plasmid is a circular DNA independent of host chromosome containing a replication origin, a proper regulatory region/promoter, an insert region to allow the target gene to be inserted, and a gene cassette for selective marker (O'Sullivan and Klaenhammer 1993; Shareck et al. 2004). Differently, integrative system is designed to integrate foreign gene into the host chromosome. Besides expressing an exogenous foreign gene, integrative cloning can also be used to disrupt undesirable genes in the host chromosome (Douglas et al. 2011).

Selective markers are important for cloning selection during gene manipulation and vector maintenances in the new host. However, commonly used antibiotic-resistant selective marker in molecular biology is not recommended in food-grade vectors because it may increase the risk of horizontal transfer of antibiotic-resistant genes to the intestinal microbiota of human and animals (Sybesma et al. 2006; Pedersen et al. 2005). The so-called dominant selection is an alternative selection method and is as efficient as antibiotic-resistant gene. It is typically based on the genes that encode rare or unusual sugar-fermenting enzymes to select and identify the dominant transformed cells in selection medium. The nsr gene which codes a hydrophobic protein was one of the first genes applied to dominant selection by resisting to nisin (Peterbauer et al. 2011; von Wright et al. 1990; Froseth and McKay 1991; Takala and Saris 2002). The α−galactosidase gene (aga) of *Lactococcus raffinolactis* (ATCC 43920) gene was demonstrated as an efficient food-grade selection marker. By comparing to traditional antibiotic selection marker (chloramphenicol-resistant gene), Labrie et al. suggested that aga gene had similar efficiency at

differentiating transformed from untransformed cells (Labrie et al. 2005). Several other resistant markers are being reported as well, including resistance to heavy metals, temperature, phage, etc. (Landete 2017).

LAB and their plasmids are increasingly attracted to the researcher and engineers for their potential applications as expression systems for the production of food-related flavorings, nutrients, as well as pharmaceutical products. Unlike other expression systems, such as viral vector, LAB and their plasmids are safer food-grade host/vector systems. Many examples of such development have been reported and reviewed (de Vos and Hugenholtz 2004; Mierau et al. 2005; Diep et al. 2009; Wells and Mercenier 2008; Son et al. 2016).

2.3.3 The Relevance to Food Industry

LAB plasmids have diverse impacts on food industry, either positive or negative. As we discussed above, LAB plasmid can help to improve product quality and quantity and provide natural or acquired resistance to phage infection for the industry. One main negative effect of plasmids to the industry is that they may trigger antibiotic resistance transfer vertically or horizontally.

Antibiotic resistance is the phenotype of bacteria to resist the inhibitory or killing effects of antibiotics (Acar and Rostel 2001; Mathur and Singh 2005). Three main horizontal gene transfers (HGTs) were reported, including conjugation, transformation, and transduction (Bennett 2008; Verraes et al. 2013). Conjugation is defined as the transfer of DNA material between bacterial cells, and a direct contact between the donor and recipient cells is required. Transformation is the process that naked plasmid DNA from the environment is directly taken by bacterial cells. The donor plasmid DNA could be released from dead or lysed bacterial cells passively or from living cells at a specific time point in their life cycle (Lorenz and Wackernagel 1994; Matsui et al. 2003; Verraes et al. 2013). Transduction is mainly a transfer process mediated by bacteriophage.

Food contamination with antibiotic-resistant genes can be found in many ways. Bacteria carrying antibiotic-resistant genes may be found in the water, soil, animal products, and fecal materials (Verraes et al. 2013). Conjugation transfer of plasmid-coded ampicillin-resistant genes from *Salmonella* to *E. coli* in milk and beef and antibiotic resistance from LAB to *Salmonella* and *E. coli* were reported (Verraes et al. 2013; Walsh et al. 2008; Toomey et al. 2009). Such antimicrobial gene transfer can also occur in the intestinal lumen of human and animals, introducing the acquired antibiotic resistance to pathogenic strains. Milk is considered as an ideal culture medium for facilitating gene transfer by conjugation and has been suggested to have ten times higher efficiency compared to laboratory culture medium (Amorim and Nascimento 2017; Verraes et al. 2013).

Food processing may involve freezing, pasteurization, UV irradiation, etc. to control the growth of or decrease the load of bacteria. These procedures may cause the release of DNA materials from stressed or damaged cells (Rajkovic et al. 2010).

Antibiotic-resistant genes that are presented in the released DNA from damaged cells may be relocated to other bacteria or pathogens and trigger gene transfer by conjugation or transformation depending on the environment. Antibiotic-resistant bacteria in the food product may be consumed and post a risk for human and animal health (Molbak 2004; Streit et al. 2006).

2.4 The Gene-Based Techniques for Rapid Identification of LAB Strains

Traditionally, most microbiologists use classical methods to examine and classify bacterial candidate. These methods include morphological observation, gram staining, and biochemical and serological analyses. The resulting taxonomy not always reflects their phylogeny and relationship by evolutionary descent. With the quick expanse of bacterial diversity, the need for new methods to discriminating those closely related bacterial strains is becoming more important. Nevertheless, the situation has swiftly changed since the introduction of PCR technology 30 years ago and quick development of sequencing technology. Accompanied with methods of molecular biology, 16S and 23S ribosomal DNA sequencing, genetic fingerprinting technologies, and fluorescent in situ hybridization (FISH) become vital for identification, classification, and functional analyses of bacterial strains.

2.4.1 16S Ribosomal DNA Sequencing and Genetic Fingerprinting

16S ribosomal RNA (16S rRNA) and 23S ribosomal RNA (23S rRNA) are the components of 30S small subunit and 50S large subunit of the bacterial ribosome, respectively. 16S rRNA genes contain highly conserved sequences between hypervariable regions (V1–V9) due to the slow rates of evolution and are frequently used in reconstructing phylogenies (Woese et al. 1990; Vetrovsky and Baldrian 2013). Compared to 16S rRNA region, 23S rRNA genes usually have higher sequence variations, such as insertions and/or deletions (Pei et al. 2009). Due to the greater length and frequent insertions/deletions, it is suggested that 23S rRNA sequencing may provide higher phylogenetic resolution and more detailed bacterial diversity (Ludwig and Schleifer 1994; Hunt et al. 2006). The 16S rRNA sequence already becomes the most popular taxonomic reference and is considered the cornerstone for modern systematic classification of bacteria (Yarza et al. 2014). As suggested by Yarza et al., lower than 94.5% of sequence identity between two 16S rRNA genes is a strong indication for distinct genera, lower than 86.5% is a strong indication for distinct families, lower than 82.0% is a strong indication for distinct orders, lower than 78.5% is a strong indication for distinct classes, and lower than 75.0% is a

strong indication for distinct phyla (Yarza et al. 2014). They also found that a reliable hierarchy of taxa requires more rRNA sequence information, and gene sequence should be longer than 1300 nucleotides.

The use of rRNA sequences for identification and phylogenetic analysis began to be accepted around 1990s (Klijn et al. 1991). DNA probes based on 16S rRNA sequences had been used to detect and identify microorganisms from samples of soil, intestinal tract, and clinical specimen, and the hybridization method with 16S rRNA probes was considered to be sensitive and efficient (Forsman et al. 1990; Rahav et al. 1990; Stahl et al. 1988). These early studies were mostly based on DNA fragment hybridization or PCR techniques and were hard to provide detailed sequence information for distinguishing bacteria on the level of strain, even on the level of species. Along with the quick development of sequencing technologies, 16S rRNA sequencing becomes a powerful tool for identification, verification, classification, quantification, and selection of bacterium or bacteria in various applications.

16S rRNA sequencing has great potential for industry application. To improve the fermentation quality of sweet sorghum, Shah and colleagues applied morphological test and 16S rRNA sequencing technique and isolated and molecularly identified five strains from 76 strains of LAB. Supplementation of these stains of LAB could enhance the fermentation quality of sweet sorghum silage (Shah et al. 2017). Lamei et al. developed a new approach for honeybee-specific LAB (hbs-LAB) identification based on sequencing of the 16S rRNA gene amplicon on the Illumina sequencing platform with an error correction software. They could rapidly distinguish and identify 13 hbs-LAB on the strain level (Lamei et al. 2017). Biogenic amine (BA) buildup in cheese is a health concern to consumers. To identify the enzyme that is responsible for BA degradation activities in LAB isolated from traditional Sicilian and Apulian cheeses, Guarcello and colleagues used PCR-based methods to complete the selection of 94 out of the 431 isolates containing genes encoding the enzyme for BAs degradation. By partial 16S rRNA sequencing, they found that 78 out of the 94 strains were *Lactobacillus* species. This study demonstrated the power of 16S rRNA sequencing in the identification of amine-oxidizing dairy LAB and the gene involved in degradation of BAs (Guarcello et al. 2016).

Genetic fingerprinting or DNA fingerprinting is a procedure of producing and analyzing of characteristic patterns of DNA fragments produced by restriction enzyme digestion. The DNA can be bacterial chromosome or PCR-amplified DNA fragments. Several molecular techniques can be considered as DNA fingerprinting, including amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), and PCR-denaturing gradient gel electrophoresis (DGGE) (Yadav and Shukla 2017).

ARDRA is modified from RFLP (restriction fragment length polymorphism) to the 16S gene. It first amplifies the 16S gene with a pair of specific primer, followed by digestion using tetracutter restriction enzyme and separated on a high-resolution agarose gel (usually 2–3% agarose). A minimum of three restriction enzymes must be used for the analysis to avoid ambiguous patterns, since certain restriction

enzymes may yield similar digestion patterns. Similar to 16S and 23S sequencing, ARDRA is more ideal for identification and differentiation of bacteria on the level of species (Sklarz et al. 2009). The first such example of using ARDRA to identify strains of bacteria at the species level was reported by Vaneechoutte et al. (1992). With similar approach, Venema et al. obtained PCR DNA fragments from the strains of 12 different bifidobacterial species from the human alimentary tract. The PCR-amplified DNA fragments were digested with five different restriction enzymes. The pattern of the differently digested fragments was applied as fingerprint to identify individual bifidobacterial species. The method was subsequently used by same research group to speciate bifidobacterial isolates from human feces and from the large intestine model in vitro (Venema and Maathuis 2003). To identify and differentiate bacteria from commercially fermented milks with bifidobacteria, ARDRA was performed on 13 *Lactobacillus*, 13 *Streptococcus*, and 13 *Bifidobacterium* strains isolated from fermented milk. The ARDRA method could discriminate these three related genera with the restriction/digestion of only one enzyme (Carmen Collado and Hernandez 2007).

RAPD is a type of PCR that amplifies bacterial genome DNA segments randomly using several arbitrary and short primers (8–12 nucleotides) to differentiate between genetically distinct individuals, although may not be in a reproducible way. The advantage of this method is that the DNA templates for PCR are the whole genome and no requirement for pre-sequencing of DNA. It is originally used in taxonomic and phylogenetic approached to study genetic polymorphisms (De Wolf et al. 2004). RAPD has been applied to study many organisms, including plants, algae, and fish. It also has been used to study the genotoxicity from various contaminants, such as cadmium, boron, arsenic, mercury, nickel, and aluminum (Baurand et al. 2015). Many studies on LAB isolates have been reported using RAPD recently; majority of these studies combined with some other molecular methods to investigate their identity, diversity, dynamics, etc. Leite et al. had identified a total of 34 LAB and characterized 150 isolates from four different Brazilian kefir grains. To exclude replicates in these isolates, they combined repetitive extragenic palindromic PCR and RAPD techniques to perform molecular typing analyses. With a threshold of 90% similarity, they reported that 32 different strains were isolated (Leite et al. 2015). In order to understand the microbial composition during sourdough fermentation of gluten-free flour at the species and strain level, Rodríguez et al. monitored autochthonous LAB microbiota using a biphasic approach combining RAPD and DGGE analyses. The strain characterization led to the isolation of *Lact. plantarum* CRL1905 and *Leuconostoc mesenteroides* CRL1907 as candidates for the functional starter culture of the gluten-free flour-fermented products (Ruiz Rodriguez et al. 2016). To select the LABs that are potentially able to arrive alive and metabolically active to the colon, Ricciardi and colleagues evaluated the dynamics of lactic microbiota of simulated digestion of cheese samples using RAPD/DGGE methods. Sixty-three strains from *Lactobacillus plantarum* and *Lactobacillus casei* groups could tolerate the condition of simulated gastrointestinal transit (Ricciardi et al. 2014). Accumulation of biogenic amines (BAs) in dairy products is a concern of public health. In order to identify the enzymatic activities responsible for BA

degradation in LAB of traditional Sicilian and Apulian cheese, 431 isolates which had no coding gene responsible for BA formation were selected using PCR-based methods. Ninety-four out of 431 of the isolates were able to degrade BAs. Further analyses by RAPD and partial sequencing of 16S rRNA, Guarcello et al. found that 78 out of 94 strains were *Lactobacillus* species (including *Lactobacillus casei*, *Lb. parabuchneri*, *Lb. fermentum*, *Lb. paracasei*, *Lb. rhamnosus*, and *Lb. paraplantarum*), *Leuconostoc* species (including *Leuconostoc lactis* and *Ln. mesenteroides*), *Pediococcus pentosaceus*, *Lactococcus lactis*, *Streptococcus* species (including *Streptococcus gallolyticus* and *S. thermophilus*), *Weissella paramesenteroides*, and *Enterococcus lactis* (Guarcello et al. 2016).

AFLP or AFLP-PCR is also a PCR-based molecular method of DNA fingerprinting. It uses restriction enzyme to digest genomic DNA and then add adaptors to the cohesive ends of the digested DNA fragments, followed by PCR amplification of these fragments using primers complementary to the sequence of annealed adaptors. The PCR-amplified DNA fragments are then separated on agarose gel electrophoresis and visualized by imaging system. Compared to RAPD, AFLP provides higher reproducibility, resolution, and sensibility at the whole genome level. Similar to RAPD, no prior sequence information is needed for the analysis. AFLP is also called as "amplified fragment length polymorphism"; the data analysis is not scored as length polymorphisms, but the presence-absence polymorphism (Vos et al. 1995). *Lactobacillus rhamnosus* is a dominant species during the fermentation of Parmigiano–Reggiano cheese and can easily adapt to unfavorable growth conditions. Bove et al. compared the isolated *L. rhamnosus* from Parmigiano–Reggiano cheese grown both in rich medium (MRS) and cheese-like medium (CB) using cDNA-AFLP. They found that the expression of a large part of *L. rhamnosus* genes modifies when cultivated in CB compared with growth under optimal conditions (MRS). The gene profiles of *L. rhamnosus* grown in CB were more diverse, probably due to the activation of alternative metabolic pathways for more energy output to respond to the environmental impacts (Bove et al. 2011). In order to study the naturally occurring LAB flora in the gut of two healthy calves (age of 65 days), Busconi and colleagues collected and cultured more than 1000 of presumptive LAB from calf gastrointestinal tracts. A total of 311 strains were analyzed and grouped into eight clusters based on their AFLP-banding patterns. Further 16S rRNA sequencing analyses revealed that the most representative genera of LAB in the isolates were *Lactobacillus* (54% of total) and *Streptococcus* (32% of total), while the most frequent species among *Lactobacillus* was *L. mucosae* with 86 different isolates (51% of the *Lactobacillus* spp. and 28% of the total) (Busconi et al. 2008). This study demonstrated that AFLP is an efficient method to characterize LAB microflora at the strain level. Ceapa and colleagues used similar approach to characterize the diversity of carbohydrate utilization capabilities of *L. rhamnosus* strains isolated from human and derived from food in relation to their niche of isolation and genotype. Genetic analyses of the strains by AFLP method suggested the occurrence of niche enrichment within particular genetic clades. Combining the high-resolution carbohydrate utilization profiling, they could establish a correlation between the carbohydrate utilization capacities and genotype/niche adaptation of *L. rhamnosus* species (Ceapa et al. 2015).

PFGE is a gel electrophoresis under pulsed-field condition to separate large DNA molecules. The method is first proposed and established by David C. Schwartz and Charles Cantor at Columbia University at 1984. Traditional agarose gel electrophoresis method is very difficult or unable to separate very large DNA molecules efficiently, usually large than 15–20 Kb. PFGE is very similar to a typical gel electrophoresis. Instead of running in one direction, the voltage of PFGE is swapped periodically in three directions by adding two additional directions at an angle of 60 degrees either side around the central axis symmetrically. A net forward migration of the DNA is achieved by equal pulse time for each direction. PFGE takes much longer running time compared to traditional gel electrophoresis, not only because of the large size of the DNA fragments being resolved but also because the DNA does not run in a straight line through the gel (Kaufmann 1998; Herschleb et al. 2007). To study the genome organization and structure of *L. gasseri* neotype strain (ATCC33323), Abs El-Osta et al. digested the bacterial chromosomal DNA with the rare-cutting restriction enzymes I-CeuI, ApaI, SmaI, CspI, and SgrAI and then subjected to PFGE analysis. The procedure and analyses let them to predict that the size of *L. gasseri* chromosome is about 1.96 Mbp. They also found the presence of a linear plasmid of 48.5 Kb in *L. gasseri* (Abs El-Osta et al. 2002). To monitor the succession dynamics of LAB populations in chill-stored vacuum-packaged beef, Jones collected drip samples from ten vacuum-packaged beef striploins stored at −1.5 °C at 4-week intervals and analyzed LAB population, pH, and spoilage-causing fermentation products. Using PFGE and analytical chemistry, he observed a gradual progression pattern during storage between strains of *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. He also found that the production of acetic acid was accompanied with the population increasing LAB generally, while the production of butyric acid was associated with a particular strain of *Leuconostoc*, and change in pH is suggested as a driving force for succession (Jones 2004). In a study of lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions, Doulgeraki and colleagues used PFGE to analyze the dynamics of the isolated LAB strains and found that packaging conditions affected *Lb. sakei* strain spoilage dynamics (Doulgeraki et al. 2010).

DGGE and temperature gradient gel electrophoresis (TGGE) are types of electrophoresis using either chemical or temperature gradient to denature the DNA samples as they move across a polyacrylamide gel. While DGGE is based on different denaturing abilities of same-size DNA determined by base-pair sequence, TGGE relies on temperature-dependent DNA structure change for separation. TGGE is a development of original DGGE method (Fischer and Lerman 1983; Rosenbaum and Riesner 1987). Most DGGE and TGGE studies have concentrated on the bacterial diversity of samples by comparing the numbers and patterns of separated bands generated on the acrylamide gel. To determine molecular diversity of *Lactobacillus* spp. and other LAB in the human intestine, Heilig et al. first selective-amplified 16S ribosomal DNA (rDNA) from lactobacilli and related LAB, including members of the genera *Leuconostoc*, *Pediococcus*, and *Weissella*. PCR amplification was performed on a variety of samples, including feces and cecum,

and analyzed by DGGE. They found that the *Lactobacillus* community in three adults over a 2-year period was different in composition and stability depending on the individual, while successional change of the community occurred during the first 5 months of an infant's life. They concluded that "the combination of specific PCR and DGGE analysis of 16S rDNA amplicons allows the diversity of important groups of bacteria that are present in low numbers in specific ecosystems to be characterized, such as the lactobacilli in the human GI tract" (Heilig et al. 2002). Evolution of the bacterial composition is crucial for the wine quality during winemaking. To study the evolution of microorganisms and for early detection of undesirable strain, Renouf and colleagues used rpoB as a target for DGGE analysis to identify LAB. They were able to analyze the microbial changes and discriminate each species during winemaking. The method also avoided the interspecies heterogeneity problem caused by 16S rRNA gene analysis only (Renouf et al. 2006). In a study of lactic acid bacteria (LABs) and yeast dynamics during the processing of sweet-leavened goods manufactured with type I sourdoughs, 14 sourdough and dough samples were taken from the production lines of three varieties of panettone. The DGGE fingerprinting of the cultivable and noncultivable microbial populations showed a dominance of *Lactobacillus sanfranciscensis*, *Lactobacillus brevis*, and *Candida humilis* in the three fermentation processes. The analysis also revealed a microbial population shift in the final stages of two of the production processes (Garofalo et al. 2008). Chinese soybean pastes were widely consumed in many countries, particularly in Asian countries. Yet, the microbial population and dynamics involved in the soybean paste fermentation process have not been studied extensively. Zhao and colleagues used DGGE method to analyze the changes in microbial community during the 12-week fermentation of natural Chinese soybean pastes. They found that *Bacillus megaterium*, *Lactobacillus plantarum*, *L. fermentum*, *B. amyloliquefaciens*, and two uncultured bacteria were predominant strains during fermentation. They also found the presence of bacteria in a less dominant way, such as *Candida humilis*, *Kluyveromyces lactis*, *Zygosaccharomyces rouxii*, and *Williopsis saturnus*. Compared to the industry starter culture which only contained *Z. rouxii* and *L. plantarum*, more microbes participated in the natural fermentation process of Chinese soybean paste (Zhao et al. 2009).

2.4.2 Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) is a technique using fluorescence-labeled DNA probes that hybridize to the parts of chromosome where a high-degree sequence complementarity is existing. It was developed by research scientist in the early 1980s and is used to detect and analyze the presence or absence of a particular DNA sequence on chromosome in cells or tissue sections (Langer-Safer et al. 1982; Thiele et al. 2012). FISH can also be used to detect RNA sequence specifically and, thereby, to analyze the expression of genes in question within cells and tissues. Combined with epifluorescence, confocal microscopy, and flow cytometry

techniques, FISH provides rapid, sensitive, and precise information about the presence, intensity, and distribution of bacteria in cells and tissues.

FISH technique has been used widely to detect specific LAB in natural or industry food products including cheeses, wine, and fish and in the human gut and faces (Machado et al. 2013; Sghir et al. 2000; Ringø et al. 2010; Harmsen et al. 1999; Vaughan et al. 2005). To directly identify and quantify LAB species in wine by microscopy, Blasco et al. used specific fluorescent oligonucleotide probes for various species belonging to the four LAB genera (*Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Oenococcus*) and hybridized to the bacteria fixed on the filters. They demonstrated that FISH method could rapidly identify LAB in wine within several hours (Blasco et al. 2003). In a study of the yeast population diversity during wine fermentations, Xufre et al. synthesized fluorescent-labeled oligonucleotide probes complemented to the D1/D2 region of the 26S rRNA of different yeast species, known to be influenced by the environment. FISH analyses showed a high diversity of non-*Saccharomyces* yeast species in white and red grape musts, including *Candida stellata*, *Hanseniaspora uvarum*, *H. guilliermondii*, *Kluyveromyces marxianus*, *K. thermotolerans*, and *Torulaspora delbrueckii*. With the FISH method, they further demonstrate that the growth advantage of *S. cerevisiae* over *Saccharomyces* yeasts only occurred after ethanol reached concentrations around 4–5% (v/v) (Xufre et al. 2006). In the commercial production of poly-L-lactic acid plastic from biomass wastes, an efficient fermentation process to produce good quantity of optically active L-lactic acid is desired. Applying FISH analyses using 16S rRNA-targeted oligonucleotide probes for *B. coagulans*, Bcoa191, and LAC722(L), Sakai and colleagues monitored the microflora in different culture condition and found that high-temperature open lactic acid fermentation which had dominant *B. coagulans* produces high-grade L-lactic acid from biomass waste (Sakai and Ezaki 2006).

2.5 Exploiting Genetic Information to Explore the Functions of LAB

The microbial community plays an important role in the verdict of our health or illness. It is well-documented that probiotics, including LAB, have great influence on the human gut microbiota. Such direct influence may only occur in the digestive system, but the indirect influence could affect not only in the gut area but the health of whole body and improve the outcome of the illness such as diarrhea, diabetes, cancer, and even dementia (Heyman 2000; Honda et al. 2012; Rajoka et al. 2017; Mehta et al. 2017).

2.5.1 LAB in the Gut

Applications of 16S rRNA sequencing and its related technologies are emerging in the fields of food-related human health, particularly human gut microbiota, probably due its fast speed of analysis, reasonable cost, and high capacity of data output. The human gut has the largest numbers of bacteria and the huge number of species, and most of them are anaerobic. Although lactobacilli have been suggested to constitute less than 1% of the bacterial community, they are predominant in the small intestine (Te Biesebeke et al. 2004).

Survival under the severe conditions in the gastrointestinal tract (GIT) is critical for LAB to exert its activities (Fuller 1989). The expression of niche-related genes may contribute to the survival of bacteria in GI transition (Azcarate-Peril et al. 2008). The active probiotics have been suggested to assist the maintenance of gut homeostasis, decrease GI transit time, regulate host metabolism, and improve epithelial barrier properties (Selle and Klaenhammer 2013). Several studies in both inflammatory bowel disease (IBD) patients and animal models suggested the impact of intestinal bacteria on the development of UC. A lower number of bifidobacteria in the feces of ulcerative colitis (UC) patients have been reported (Saez-Lara et al. 2015). Indeed, supplementation of bifidobacteria-fermented milk (BFM) may slow the development of UC by normalizing the intestinal microbiota (Tursi et al. 2010). *Lactobacillus plantarum* ZS2058 produces conjugated linoleic acid (CLA) in culture. In order to study its function in vivo, *Lactobacillus plantarum* ZS2058 was tested in a dextran sodium sulfate-induced acute colitis mouse model. Compared to *L. plantarum* ST-III which do not produce CLA, *L. plantarum* ZS2058 significantly lowered the disease activity index (DAI) and inhibited colon shortening and myeloperoxidase activity in colitic mice. Treatment of *L. plantarum* ZS2058 also improved the histological damage and protected the colonic mucous layer integrity. Cytokine analyses which showed the treatment also significantly attenuated the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and upregulated the expression of colonic anti-inflammatory cytokine IL-10. CLA concentration in the colon was significantly increased in response to *L. plantarum* ZS2058 treatment, indicating that ZS2058 exerted its protective effects by producing CLA in the colon (Wang et al. 2016).

Foodborne pathogens cause many public health problems. Several studies have evaluated the effect of LAB on preventing *Salmonella* infection. Campana et al. examined seven LAB strains for antimicrobial activities and interference studies against five human intestinal pathogens (*Salmonella* Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* O157: H7 ATCC 35150, *Cronobacter sakazakii* ATCC 29544, and *Campylobacter jejuni* ATCC 33291). They found that the strains showed some antimicrobial activities in vitro and strain-specific abilities to reduce the invasion of intestinal pathogens in Caco-2 cell culture model (Campana et al. 2017). Studies showed that the management of *L. acidophi-*

lus LB increased eradication rate of *H. pylori* from 72 to 87% (P<0.01), and supplementation with *Lactobacillus rhamnosus* GG reduced bloating, taste disorder, and diarrhea associated with an *H. pylori* eradication (Kafshdooz et al. 2017). However, Lee et al. reported that there were no significant inhibitive effects on *H. pylori* when they tested three probiotic strains (*L. acidophilus*, *L. rhamnosus*, and *L. sporogenes*) in a human trial (Lee et al. 2017).

2.5.2 LAB as Probiotics for Human Health

Beneficial effects of LAB are not only limited to the health of our digestive system but also outer of our digestive system, including diabetes, obesity, hyperlipidemia, cancer, and dementia. A recent study reported that colonization of gut microbiota can affect adult behavior and mammalian brain development. The study suggested that the process of such colonization triggered signaling machineries that regulated neuronal circuits of anxiety behavior and motor control (Heijtz et al. 2011). Similarly, several key studies indicated that alteration in gut microbial population could noticeably affect central physiology, transfer the gut microbiota from animal to animal, and transfer the behavioral or physiological phenotype (Dinan and Cryan 2017). The favorable properties of LAB on human health are numerous; only several pronounced beneficial effects of LAB as a probiotics for human health will be discussed below.

Several clinical trials have studied the effects of LAB on diabetes management. Karamali et al. initiated a clinical trial to study the effects of probiotic supplementation on the levels of glucose and lipids in patients with gestational diabetes mellitus (GDM). Sixty pregnant GDM patients between ages 18 and 40 received the capsules of either probiotic or placebo for 6 weeks. The probiotic capsule contained *Lactobacillus acidophilus*, *L. casei*, and *Bifidobacterium bifidum*. The daily dosages were 2 × 109 CFU/g for each strain. Compared to placebo, probiotic supplementation resulted in significant decreases in fasting plasma glucose (−9.2 ± 9.2 mg/dL vs. +1.1 ± 12.2 mg/dL, $P < 0.001$), serum insulin levels (−0.8 ± 3.1μIU/mL vs. +4.5 ± 10.6μIU/mL, $P = 0.01$), insulin resistance (−0.4 ± 0.9 vs. +1.1 ± 2.5, $P = 0.003$) and β-cell function (+1.1 ± 9.8 vs. +18.0 ± 42.5, $P = 0.03$), significant increase in the insulin sensitivity (+0.007 ± 0.01 vs. −0.01 ± 0.02, $P = 0.007$), and significant improvement of lipid profile, including lower triglycerides (−1.6 ± 59.4 mg/dL vs. +27.1 ± 37.9 mg/dL, $P = 0.03$) and higher VLDL cholesterol concentrations (−0.3 ± 11.9 mg/dL vs. +5.4 ± 7.6 mg/dL, $P = 0.03$). These results demonstrated that probiotic supplementation in patients with GDM had improved patient conditions, including glucose, triglycerides, and VLDL cholesterol levels (Karamali et al. 2016). Tajabadi-Ebrahimi and colleagues designed a randomized trial to investigate the effect of synbiotic administration with type 2 diabetic overweight patients with coronary heart disease. Patients were divided into two groups (n = 30), and each patient received either synbiotic supplements containing three probiotic bacterial species, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* (2 × 109 CFU each species), or 800 mg inulin or placebo

for 12 weeks. Consumption of synbiotic capsule significantly decreased plasma glucose level (− 19.6 ± 74.6 vs. + 19.2 ± 66.9 mg/dL, $P = 0.03$), serum insulin concentrations (− 0.7 ± 5.1 vs. + 3.3 ± 6.3 μIU/mL, $P = 0.01$), B-cell function assessed by the homeostasis model (− 3.4 ± 19.5 vs. + 11.5 ± 21.0, $P = 0.006$), insulin sensitivity index (+ 0.002 ± 0.01 vs.−0.01 ± 0.02, $P = 0.03$), and HLDL levels (+ 1.8 ± 5.7 vs.−2.2 ± 6.0 mg/dL, $P = 0.01$) compared with the placebo. They concluded that supplementation of synbiotic had beneficial effects on insulin and cholesterol regulation for diabetic patients with CHD (Tajabadi-Ebrahimi et al. 2017). Another randomized trial studied the effects of *Lactobacillus rhamnosus* HN001 on early pregnant woman with gestational diabetes mellitus (GDM). The study found that HN001 supplementation for GDM patients from 14 to 16 weeks' gestation reduced GDM incidence, particularly among older women and those who previously had GDM (Wickens et al. 2017).

LAB supplementation also has been used for clinical trial for cancer therapy, mostly combined with other therapeutic agents or procedures. One randomized double-blind trial studied the effects of prebiotic and synbiotic treatment on patients before colorectal surgery. Seventy-three patients with preceding colorectal operations were divided into three groups randomly. The first group received prebiotics preoperatively, the second synbiotics in, and third was preoperatively cleansed. The study did not find any significant differences in systemic inflammatory response and no differences in complication rate and postoperative course among all three study groups (Krebs 2016). Kotzampassi et al. conducted a clinical trial to evaluate the efficacy of a new probiotic formulation as prophylaxis for postoperative complications after colorectal surgery. A combination of four probiotic strains was formulated into the capsules, including *Lactobacillus acidophilus*, *L. plantarum*, *Saccharomyces boulardii*, and *Bifidobacterium lactis*, and supplemented 1 day before operation and continued for 15 days postoperatively. The study found that supplementation of probiotics had significant effects on decreasing the rate of all postoperative major complications (28.6 vs. 48.8% of the placebo arm, p 0.010, odds ratio 0.42), and major beneficial effects were found in the reduction of postoperative pneumonia rate (2.4 vs. 11.3%, p 0.029), surgical site infections rate (7.1 vs. 20.0%, p 0.020), and anastomotic leakage rate (1.2 vs. 8.8%, p 0.031). The study concluded that supplementation of probiotic could significantly decrease the risk of postoperative complications (Tsaousi et al. 2017). Chemoradiotherapy for head and neck squamous cell could cause frequent and serious complication of oral mucositis. Sharma et al. evaluated the effects of supplementing *Lactobacillus brevis* CD2 lozenges on the frequency and severity of mucositis and chemoradiotherapy tolerance. Two hundred patients were included in a double-blind study by daily supplementation of lozenges containing either *L. brevis* CD2 or placebo. The efficacy analysis showed a decline in Grades III and IV mucositis (52% with *L. brevis* CD2 vs. 77% with placebo, $P < 0.001$), an increase in anticancer treatment completion rate (92% with *L. brevis* CD2 vs. 70% with placebo, $P = 0.001$), and a higher rate of patients remained free of mucositis (28% with *L. brevis* CD2 vs.7% with the placebo). The study concluded that *L. brevis* CD2 reduced the incidence of anticancer therapy-induced oral mucositis and increased the rate of anticancer treatment completion (Sharma et al. 2012).

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Chapter 3
Proteins and Exopolysaccharides of Lactic Acid Bacteria

Haiqin Chen and Arjan Narbad

Contents

Abstract Bacteriocin is peptide produced by bacteria to inhibit or kill other bacteria, exopolysaccharides are long chain polysaccharides composed of repeating sugar units. In the past decade, interest in bacteriocin and polysaccharides research from lactic acid bacteria have obtained great momentum due to their potential functions. This chapter will summarize current literature on the biological characteristics and functions of protein and exopolysaccharide produced by lactic acid bacteria, and discuss their potential applications.

Keywords Bacteriocin · Exopolysaccharide · Classification · Chemical structure · Functionality

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https://doi.org/10.1007/978-981-13-1559-6_3

3.1 Introduction

This chapter will summarize the current literature on the biological characteristics and functions of protein and exopolysaccharide produced by lactic acid bacteria and discuss their potential applications.

3.2 Bacteriocins

Bacteriocin is a ribosomally synthesized peptide produced by bacteria to inhibit or kill other bacteria in competition for nutrients or habitats. In the past decade, interest in bacteriocin research, especially lactic acid bacteria (LAB), has obtained great momentum due to its potential function both as a natural food preservative and therapeutic antibiotics (Cotter et al. 2005a; Van Heel et al. 2011). Bacteriocin has a number of advantages over conventional antibiotics. The spread of antibiotic-resistant bacteria poses a great threat to public health and is growing worse since the current progress in developing of novel antibiotics is limited (Brown and Wright 2016). There is a requirement for new antimicrobials that can be used as alternatives to conventional antibiotics.

3.2.1 The Classification and Chemical Structure of Bacteriocins

3.2.1.1 The Classification of Bacteriocins

The classification previously established by Klaenhammer grouped bacteriocins into four distinct classes with further subclasses. Before this classification, there were several classification approaches of bacteriocin. For example, early classification methods based on LAB bacteriocins' characteristics classified the individual bacteriocins into eight groups, such as host range, host combinations, trypsin sensitivity, heat resistance, and the degree of cross-reactivity between various bacteriocins (Drider and Drider 2011a). After Klaenhammer, reclassifications or adaptations were performed by van Belkum and Stiles (2000) and Abriouel H et al. (2010) for the enterocin bacteriocins. In addition, the Cotter proposal has just two major categories: the lantibiotics (Class I) and the non-lantibiotic bacteriocins (Class II). As recently recommended by Kemperman et al. (Riley and Wertz 2002), Klaenhammer's classes IIc and IV were canceled, since the two classes are unproven entities and they have novel posttranslationally modified cyclic peptides (Tagg et al. 1976; Riley and Wertz 2002). As assigned by Klaenhammer, Class IV was relegated to Class IIc. Heng NCK et al. have proposed a classification scheme, which is in line with Cotter et al. The Klaenhammer classes IIc and IV were eliminated. However, reverse to Cotter, Class III (large bacteriocins) is reserved and separated into Types IIIa (bacteriolytic) and IIIb (non-lytic), and the cyclic posttranslationally modified

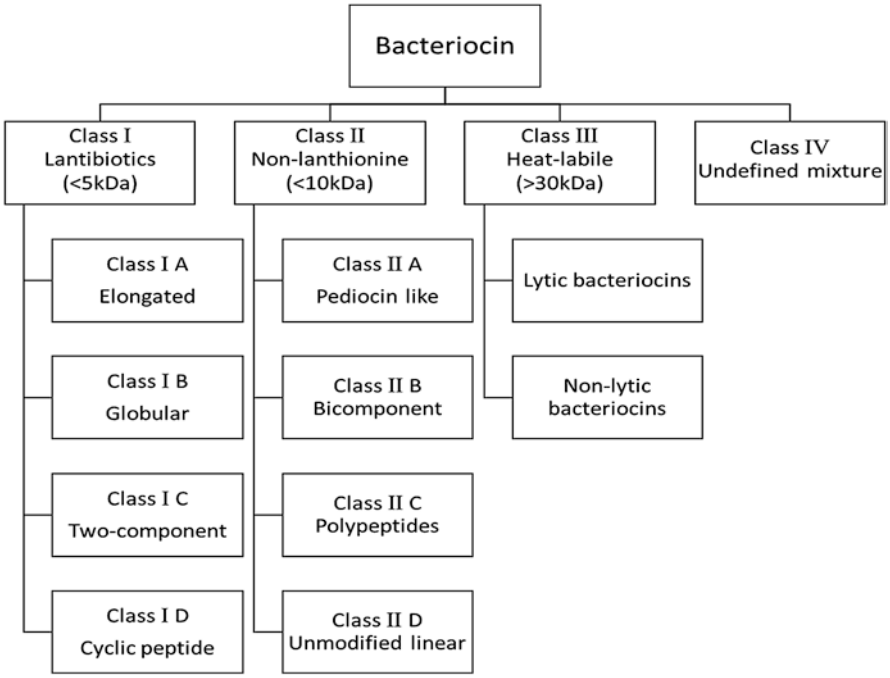

Fig. 3.1 The classification of bacteriocin. (Ahmad et al. 2017)

bacteriocins (Class IIc) are upgraded to Class IV (Heng and Tagg 2006). They believe that this classification scheme can be applied into most bacteriocins, if not all, disregarding the Gram status of the producer strain. And they acknowledged that this scheme will be continually completed as the development of our knowledge of microbial diversity (Ahmad et al. 2017).

The classification can be summarized as follows (Fig. 3.1):

Class I: Class I is named as lantibiotics, because these bacteriocins have one common characteristic that they all contain lanthionine, and nisin is the most typical bacteriocin in the Class I bacteriocins. The relative molecular mass of this category is less than 50,000, including more than 19–38 amino acid residues. Such bacteriocin-active site contains a lot of rare amino acid molecules, including lanthionine (Lan), β-methylmethionine (MeLan), dehydroalanine (Dha), and dehydrobutyrine (Dhb), and other noncoding amino acids.

Class I can be divided into four subcategories: type A is elongated, with positive charge and amphipathicity, which can form potential dependence holes in bacterial plasmids, like subtilin, ericin S, and ericin A. Type B is globular, without charge or negative charge, mainly through destroying enzymes' function to work. Type B also includes other lantibiotics such as paenibacillin and sublancin 168. Type C is a two-component lantibiotics, which contains two polypeptide chains, and they are synergistic with each other to act as bacteriostatic agent, such as lichenicidin and haloduracin. Type D includes the unique cyclic peptide subtilosin A that contains a head-to-tail peptide bond as well as special sulfide bridges formed between cysteine groups and dehydrated amino acid residues (Abriouel et al. 2011).

Class II: defined as small-molecule (relative molecular weight < 10kDA), thermal-stable, and membranous-active polypeptide, non-lanthionine, such as tablet (pediocin pa-1). These were further subdivided into four subgroups:

Type IIa class bacteriocin is the largest subclass of Class II bacteriocin. All of the type IIa bacteriocins discovered so far have strong resistance activity to Liszt; therefore, it was also called class piece of *Staphylococcus aureus*, also known as anti-Lister active polypeptide.

Type IIb bacteriocin is a kind of bicomponent bacteriocin, which is formed by two peptide oligomers and requires the interaction of two peptides to produce a complete activity. Type IIb bacteriocin can be divided into two categories: one is the cooperative type (S type), and the other is the enhanced type (E type). Lactococcin Q, a new two-peptide bacteriocin from an LAB called *L. lactis* QU isolated from corn, is comprised of peptides Qa and Qb (Zendo et al. 2006). These two peptides have showed high comparability to two peptides Ga and Gb, which comprise the bacteriocin lactococcin G (Nissen-Meyer et al. 1992). The antimicrobial spectra of lactococcins G and Q are very specific, and they only have antimicrobial activity for strains derived from *L. lactis*.

Type IIc bacteriocin, including those who belong to neither type IIa class nor type IIb non-lantibiotics, is thought to be polypeptides containing the activity of sulfhydryl and the signaling peptide encoding mechanism. The bacterial strains of type IIc are diverse; therefore, the species diversity of this kind of bacteria is also more complex (Cotter et al. 2005a).

Type IId: linear, unmodified, non-pediocin-like bacteriocins. This type of bacteriocins is typically considered to be synthesized without an N-terminal leader sequence. Leaderless bacteriocins are often relatively small, usually 30–50 amino acids, and they do not like other bacteriocins go through posttranslational modifications. Therefore, they contain an N-terminal formylmethionine (Patricia et al. 2016). These characteristics make them relatively easy to obtain through synthetic methods, which opens up new possibilities for detailed bacteriocin research and design of new peptides with improved properties (Ovchinnikov et al. 2014).

In general, these peptides show broad-spectrum activity against Gram-positive bacteria. And occasionally, if the outer membrane of the bacteria is destroyed, they also can show some activity in Gram-negative bacteria (Towle and Vederas 2017).

Class III: bacteriocins in this class (formerly Class III bacteriocins) are large, heat-labile antimicrobial proteins. The relative molecular weight of Class III bacteriocin was found to be larger (usually greater than 30 kDa), and the bacteriocin is thermolabile; it is usually inactive in less than 30 min of 100 °C heating. Such bacteriocins can also be subdivided into two categories: one is lysozyme (lytic bacteriocins) that works through dissolving the cell, and the other is antibacterial protein (non-lytic bacteriocins) (Joerger and Klaenhammer 1986). Enterolysin A cleaves within the peptidoglycan of target cells between L-alanine and D-glutamic acid of the stem peptide and between L-lysine of the stem peptide and D-aspartic acid of the interpeptide bridge. Not only lytic bacteriocins but also some heat-labile, high-molecular-weight bacteriocins without a lytic mode of action have also been found, such as helveticin J from *Lactobacillus helveticus* 481, dysgalacticin from

Streptococcus dysgalactiae subsp. *equisimilis* W2580, and streptococcin A-M57 (Vaughan et al. 1992; Eijsink et al. 1998). The action mode of dysgalacticin has been studied, and it has been determined that this bacteriocin interferes with glucose transport or metabolism by conjoining the phosphoenolpyruvate-dependent glucose or mannose phosphotransferase transport system (Eijsink et al. 1998).

Class IV: the fourth type of bacteriocin consists of an indeterminate constituent, a mixture of proteins, lipids, and carbohydrates. The existence of the fourth category is mainly supported by the observation that some bacteriocin activities (e.g., Lb. plantarum LPCO10) are obtained in cell-free supernatants, which are not only eliminated by protease treatment but also by glycolysis and lipolysis enzyme. They cannot only restrain Gram-positive bacteria but also have inhibition effects to Gram-negative bacteria and fungi. Therefore, antagonistic substances that do not meet or completely meet the bacterial protein definition are called bacteriocin-like substance. AS-48 (a circular bacteriocin) produced by *Enterococcus faecalis* is classified into this new class (Sánchezhidalgo et al. 2011a). AS-48 has attracted much attention due to its broad spectrum against Gram-positive bacteria including *Clostridium tyrobutyricum*, *Enterococcus faecalis*, *Listeria monocytogenes*, and some strains of *Staphylococcus aureus* (Towle and Vederas 2017).

Ahmad Cheikhyoussef, Wei Chen, and Hao Zhang et al. presented a review of the antimicrobial proteinaceous compounds produced by various *Bifidobacterium* strains, including bifidin (*B. bifidum*), biflong (*B. longum*), bifidocin B (*B. bifidum* NCFB 1454), bifilact Bb-12, bifilong Bb-46, etc., and summarized the identification, characterization, and possible applications of these compounds (Cheikhyoussef et al. 2008). Antimicrobial activity spectrum of the proteinaceous inhibitory compounds obtained from bifidobacteria can be summarized in Table 3.1.

Four strains of *Bifidobacterium* showed different degrees of antagonistic action toward the indicator strain. The maximum degree of inhibition was from *Bifidobacterium infantis* (96%) and *Bifidobacterium longum* (92%). We found that substances or factors other than sole organic acids may contribute to the antimicrobial activity of the supernatants from the bifidobacteria studied (Cheikhyoussef et al. 2007).

In our laboratory, *Bifidobacterium* infants BCRC 14602 was found to produce a bacteriocin-like inhibitory substance (BLIS), which have a wide antimicrobial spectrum against Gram-positive and Gram-negative bacteria. Firstly, the purification of BLIS has been studied. BLIS was partially purified by a two-step purification scheme resulting in a specific activity of 31,605 AU/mg and a purification fold of 120. Then, a series of characteristics of BCRC 14602 BLIS were recognized. The size of BLIS is approximately 3.0 kDa based on tricine–SDS–PAGE, and it is sensitive to proteolytic enzymes but insensitive to catalase, lipase, and α-amylase. BLIS has high temperature stability and high pH stability in the range of 4–10. The adsorption of BLIS to production cells is strongly influenced by the pH of the broth culture that occurs 100% adsorption on dead cells between pH 6.0 and 7.0 (Cheikhyoussef et al. 2009).

In addition to BLIS, a new bacteriocin bifidin I from *Bifidobacterium infantis* BCRC 14602 was studied. Similar methods were used to get the characteristics of bifidin I. Unlike BLIS, the purification process of bifidin I was more complicated,

Table 3.1 Antimicrobial activity spectrum of the proteinaceous inhibitory compounds obtained from bifidobacteria (Cheikhyoussef et al. 2008, 2009)

Species	Inhibitory spectrum	Reference
Bifidobacterium (dairy product isolate)	Some species of *Bifidobacterium*, *Lactococcus*, *Clostridium*, and *Lactobacillus*	Meghrous et al.
B. breve 4, *B. infantis* 1	Enteropathogenic *E. coli*, *Yersinia pseudotuberculosis*, *Salmonella typhimurium*	Bernet-Camard et al.
B. infantis	*Escherichia coli* and *Clostridium*	Gibson and Wang
B. bifidum	*Clostridium perfringens*, *Salmonella* ssp.	
B. adolescentis	*Campylobacter* ssp.	
B. bifidum	*Shigella dysenteriae*	Misra and Kuila
B. infantis	*Yersinia enterocolitica*	Ozbas and Aytac
B. longum SBT 2928	Enterotoxigenic *Escherichia coli* Pbl76 (ETEC)	Fujiwara et al.
B. animalis	*Salmonella enteritidis*	Bielecka et al.
B. bifidum NCFB 1454 (crude bifidocin B)	*Listeria*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*	Yildirim and Johnson
Bifidobacterium CA1 and F9 (human infant feces isolates)	*Salmonella typhimurium* SL 1344	Lievin et al.
B. lactis DR 10, *B. lactis* LKM512	Enterotoxigenic *E.coli* O157:H7 *Clostridium perfringens*	Gopal et al.; Matsumoto et al.
B. adolescentis, *B. bifidum*, and *B. longum*	*E. faecalis*, *S. typhimurium*, *C. sporogenes*, *B. cereus*, and *L. monocytogenes*	Bevilacqua et al.
B. adolescentis (RBL85), *B. longum* (RBL67), *B. bifidum* (RBL68, 69, 70, 86)	*Listeria monocytogenes*	Toure et al.
B. infantis	*Clostridium difficile* ATCC 9689	Lee et al.
B. bifidum RBL 71 and *B. bifidum* RBL 460	*E. coli* O157:H7	Gagnon et al.
BIR-0321 and BIR-0307 (human feces isolate)	*Listeria innocua* ATCC 33090, *Escherichia coli* NCTC 8603, *Salmonella typhimurium* ATCC 29631	Collado et al.
BIR-0307, BIR-0312, and BIR-0324	*Helicobacter pylori*	Collado et al.
B. longum CIDCA 5320, 5323; *B. bifidum* CIDCA 5311; *B. breve* CIDCA 532	*Clostridium difficile* ATCC 9689 and ATCC 43593	Trejo et al.
B. thermacidophilum RBL 71	*Enterohemorrhagic E.coli* O157:H7	Gagnon et al.
B. thermacidophilum and *B. thermophilum*	*Listeria monocytogenes*	Moroni et al.
B. longum and *B. infantis*	*B. cereus* and *E. coli*	Cheikhyoussef et al.
B. thermophilum subsp. *infantis* RBL 67	*Listeria monocytogenes*	Naghmouchi et al.

in which three steps were needed. The purification scheme resulted in a purification fold of 1390 with a specific activity of 365,714 AU/mg and a yield of 25.6%. We can know that the mass of bifidin I is approximately 2879.7 Da through mass spectrometry analysis. Bifidin I was recovered by adsorption–desorption onto/from silicic acid (ADSA). The pH of the broth culture strongly influences the adsorption of the bifidin I to silicic acid (5%), and 100% adsorption occurs between pH 5.0 and 7.0; otherwise the adsorption ratio decreased from 67% to 45%. Only part of NH2-terminal amino acid sequence consisting eight amino acid residues was obtained: NH2-Lys-Tyr-Gly-Ser-Val-Pro-Leu-Gly. Curing experiments which resulted in Bif variants incapable of producing bifidin I but retaining immunity indicated that production of bifidin I is plasmid associated (Cheikhyoussef et al. 2010).

Another novel bacteriocin studied in our laboratory is sakacin P which is from *Lactobacillus sakei* and showed strong anti-activity against foodborne pathogens such as *Listeria monocytogenes*. And the genetic studies were carried out. In *L. sakei*, the structural gene (sppA) encoding sakacin P is commanded by a strict regulatory mechanism, and the amount of sakacin P secreted is limited. To obtain much more sakacin P, the sppA gene was transformed into *E. coli* and inducted with isopropyl-β-D-thiogalactopyranoside. Finally, the recombinant sakacin P was successfully expressed. Through agar diffusion method, the activity of sakacin P can be improved in a low temperature (Chen et al. 2012a).

Another bacteriocin-soluble NB-C1 fusion protein has been successfully obtained in the *E. coli* cell-free protein synthesis system. The NB-C1 gene is a novel potential Class IIa bacteriocin gene with the characteristic of YGNGVxC cluster. We built the expression vector pIVEX2.4d- GFP-NB-C1 both in the continuous exchange cell-free (CECF) systems and batch mode. The amount of soluble fusion protein attained from the CECF system was 2.2 mg/ml, which is around 3 times higher than that in the batch mode (0.73 mg/ml). The soluble fusion protein was purified via Ni–NTA affinity chromatography, resulting in a concentration of 0.26 mg/ml and a purity of 95%. The purified NB-C1 fusion protein was proved to have a strong inhibitory effect on the growth of *L. monocytogenes* (Chen et al. 2012b).

3.2.1.2 The Chemical Structure of Bacteriocins

The primary structures of lantibiotics and sactibiotics have been identified by some strategies and techniques. A major challenge we need to solve is to identify the topology of thioether bridges in these peptides (i.e., which amino acid residues are involved in which bridges). NMR spectroscopy, Edman degradation, and tandem MS have all been commonly used to characterize these bacteriocins, but they are antipathic with the posttranslational modification protein. Some chemical modifications, such as reduction and desulfurization, can assist the treated bacteriocins more compatible to these standard peptide analytical techniques (Perez et al. 2014).

A rapid system for screening novel bacteriocins from various sources has been developed in the early stages of screening and isolation of bacteriocin-producing

strains. This system uses electrospray ionization liquid chromatography/mass spectrometry (ESI-LC/MS) combined with principal component analysis (PCA) to perform molecular weight analysis on the antimicrobial activity spectrum of each bacteriocin produced by the LAB strain. This allows us to save time, energy, and money and quickly track the discovery of more novel bacteriocins.

Class I: Class I bacteriocins or lantibiotics (lanthionine-containing antibiotics) are small peptides (<5 kDa) that contain abnormal posttranslational modifications such as lanthionine or 3-methyllanthionine. These residues are capable of forming covalent bonds between amino acids, forming an internal ring structure and giving unique structural features (Chen et al. 2012b; Cotter et al. 2005b). Bacteriocins are usually synthesized as non-bioactive prepeptides, including N-terminal precursor peptides connected to C-terminal peptides (And and Hoover 2003; Klaenhammer 1993; Cotter et al. 2005b).

Class II: Bacteriocin type IIa class typically contains 37–48 amino acid residues, and the N-terminal consists of conservative YGNGVXaaC groups, which is usually thought to be membrane receptor-binding protein recognition sequence. As more and more new type IIa class of bacteriocin was found, the N-terminal groups can also be shown as YGNGVXaaCXaa XaaXaaCXaaV (K/N) (N/D) (W/R/K) Xaa (G/A/S) (A/N) (the content in the parentheses is conservative residue, meaning the residue can be replaced; Xaa means high change frequency residues).

Another important feature of type IIa class bacteriocin is that N-terminal conservative contains at least two cysteines that formed disulfide bond with each other. The existence of the disulfide bond allows a hydrophilic oleophilic structure in the N-terminal, which is necessary for antimicrobial activity. In general, peptide fragment that contains two disulfide bonds has a wider bacteriostatic spectrum than peptide fragment that contains only one disulfide bond (Eijsink et al. 1998). Compared with the N-terminal conservative property, the similarity of C-terminal sequence is only 34–80%, and an α-helix is formed. The spiral structure served as cross-membrane component when holes are formed on the sensitive bacteria cell membranes.

Enterococcus faecium NKR-5-3, an LAB isolated from the Thai fermented fish (Himeno et al. 2012; Naoki et al. 2012), can produce a kind of bacteriocin named enterocin NKR-5-3C (Ent53C). Ent53C is a typical Class IIa bacteriocin, which displayed extremely strong antimicrobial activity (in nanomolar range) against *Listeria* spp. and other Gram-positive species (Drider et al. 2006). The number of disulfide bridges in Class IIa bacteriocins directly relates with the intensity of their antimicrobial activity and stability (Drider et al. 2006; Richard et al. 2006).

Class IIb bacteriocins are two-peptide bacteriocins that require the cooperation of two peptides to be fully activated (Oppegård et al. 2007; Nissenmeyer et al. 2010). The novel two-peptide bacteriocin *Lactococcus Q*, isolated from *Lactococcus lactis QU4*, a lactis isolated from corn, consists of two peptides: Qa and Qb. Structural analysis of the *Lactococcus Q* peptide showed it has the same position of α-helical structure as *Lactococcus G*. This suggested that these bacteriocins have similar modes of action (Zendo et al. 2006).

The cyclic bacteriocins (Class IIc) are characterized by their unique structural feature of a head-to-tail cyclization of their backbones (Maqueda et al. 2008; van Belkum et al. 2011). The circular bacteriocins compared to their linear counterparts usually have superior structural stability, greater thermal stress resistance, and larger stability against proteolytic digestion (Conlan et al. 2011; Craik et al. 2010), which are mainly determined by the nature of their structure. The N- and C-termini of Class IIc bacteriocins are covalently linked giving the peptide an extremely stable structure (Maqueda et al. 2008; van Belkum et al. 2011).

There are some similar structures in Class IIc bacteriocin, such as leader peptide with GG sequence, ABC transport protein, and some related immune protein. Some Class IIc bacteriocins have no identifiable N-terminal signal peptide, such as enterocin Q from excrement *Enterococcus*, aureocin A53 produced by *Streptomyces aureus*, and BHT-B produced by *S. rattus*. Bacteriocin gassercin A isolated from LA39 strains of lactobacillus in human baby feces, which contains a rare loop structure, has inhibitory activity on many food pathogenic bacteria, including *Listeria*, waxy *Bacillus*, and *Staphylococcus aureus*.

Both lactocyclicin Q (LycQ) and leucocyclicin Q (LcyQ) are circular bacteriocins composed of 61 amino acid residues (Sawa et al. 2009; Masuda et al. 2011), and their precursor peptides contain a leader sequence of two amino acid residues in which cyclization occurs between L3 and W63 (Masuda et al. 2011). The prediction of the secondary structure of the two bacteriocins reveals that the four identical α-helices, each with subtle amphiphilic properties, are thought to play an important role in their antimicrobial action.

On the other hand, Class IId bacteriocins contain the remaining bacteriocins, which bind in the wrong combination or bind as one-peptide non-pediocin linear groups (Cotter et al. 2005b). Sec-dependent bacteriocins (Cintas et al. 2000) and leaderless bacteriocins (Fujita et al. 2007) belong to this class. Lacticins Q and Z only have differences in three amino acid residues at the positions of 10, 33, and 44. In addition, they both have a formylated methionine at the starting residue. They are both 53 amino acid highly cationic peptides, exhibiting extremely strong antimicrobial activity (at nanomolar concentrations) and high stability against various stresses (Fujita et al. 2007; Iwatani et al. 2007). The antibacterial activity of lacticin Q is mainly attributed to the two amphiphilic helices, which play a major role in it (Yoneyama et al. 2009a, b, 2011). Since the homology of these two leaderless bacteriocins is very high and their activity spectra are comparable, it was inferred that they have the same mode of antimicrobial action (Iwatani et al. 2007).

Class III: Class III bacteriocins are heat-labile and usually consist of different domains. For example, on the basis of sequence analysis, enterolysin A comprises an N-terminal endopeptidase domain and a C-terminal substrate recognition domain, which is similar to zoocin A (Lai et al. 2002; Nilsen et al. 2003). The zif gene encoding the immune protein, which is close to zooA, adds L-alanine into the cross bridges of peptidoglycan, reducing the ability of animal protein A to degrade the polysaccharide layer (O'Rourke et al. 2009).

Class IV: circular and linear leaderless bacteriocins whose backbone structure assumes a saposin-like fold or a related α-helical bundle in the context of their possible mechanisms and their interactions with lipids.

3.2.2 The Genetics and Regulation of Bacteriocins

Lactic acid bacteria will secrete some bacteriocins in the logarithmic phase and transport bacteriocin to the external medium by the cell membrane permeability, but some types of bacteriocin will stay in cells under certain conditions. In general, the production and synthesis of bacteriocin is synchronized with growth, and the yield is closely related to the number of producing bacteria. The optimization of culture conditions can effectively increase the production of bacteriocin, such as the supplement of sugar, vitamin, nitrogen source, or stimulating factors such as pH, temperature to their best range, etc. (Abbasiliasi et al. 2011; Espeche et al. 2014). The pH of medium is one of the important factors which influences the production of bacteriocin, but the requirement of pH of different kinds of bacteriocin are not identical (Arauz et al. 2012).

According to Drider et al. (2006), at least four key genes are contributed to the secretion and production of bacteriocins. In particular, they are (i) the structural bacteriocin gene, encoding a prebacteriocin; (ii) an immune gene that protects bacteriocin producers from their own bacteriocins; (iii) a gene encoding an ABC transporter necessary for secretion; and (iv) a gene encoding an accessory protein of unknown function (Sabo et al. 2014).

Bacteriocin biosynthesis was regulated by the regulation genes; these genes are generally located on the chromosome or plasmid (Nes et al. 1996). Sulfide antibiotic bacteriocin and Class II bacteriocin operon are mostly located on chromosome, such as bacteriocin plantaricins EF and NC8 produced by plant lactobacillus 8p-A3. These two kinds of bacteriocin production regulation genes are located on chromosome, and the gene fragment size is about 20 kb. Besides these gene cluster located on chromosome, some gene clusters regulating bacteriocin production are located on plasmid. Mandal reported a new bacteriocin pediocin NV5 produced by lactic acid piece *Staphylococcus aureus* LAB5 whose gene cluster in a size of about 5 KB was proved to be located on plasmid by eliminated plasmid experiments, etc. (Mandal et al. 2011).

Bacteriocins' expression needs at least two genes, structure gene and immune genes. The former codes express precursor peptide, while the latter encode some immune protein to protect bacteria from the attack on its own. The bacteria have no specific output protein for bacteriocin. The bacteriocin outputs the cells through the normal output pathway, such as bacteriocin 31 produced by *Enterococcus* YI717, whose structure genes and immune genes were BacA and BacB, respectively. These gene clusters were on removable plasmid pYI17, and the size is about 57.5 KB (Tomita et al. 1996).

Bacterial structural gene encoding a bacteriocin containing N-terminal leader sequence (referred to as double-glycine leader sequence) of the preform element, which appears to function (a) preventing the bacteriocin from being biologically active when detained inside the producer and (b) providing the identification signal used in the transportation system. The length of the bio-glycine leader ranges from 14 residues to about 30 residues (Klaenhammer 1993).

Common elements found in the double-glycine leader sequence include two glycine residues at the C-terminus of the cleavage site, conserved hydrophobic and hydrophilic residues separated by distances determined in the reserved residues. In addition, the minimum length of the double-glycine leader sequence appears to be 14 amino acids. Of the consensus residues of the leader sequence, only the glycine residue at position 2 is completely conserved. The mature bacteriocins identified so far range in size from less than 30 residues to more than 100 residues (Nes et al. 1996).

Class I: Genes involved in lantibiotic synthesis are generally settled in clusters, which can be organized on a transposon (nisin), on a plasmid (epidermin), or on the chromosome (subtilin) (Drider and Drider 2011b). As for the biosynthesis of lantibiotic, it usually includes the following steps. Firstly, the process starts with translation of a leader and modifiable propeptide moiety, and then the prepeptide undergoes modification. Next, the prepeptide is translocated through the cytoplasmic membrane, and the leader peptide is cleaved proteolytically. Genes coding immunity proteins are normally sited in a cluster around the bacteriocin structural gene. The conserved sequences of PTMs and core peptides facilitate high-level analysis by analyzing the genomic data. This can help centralize screening efforts to discover new molecules using different alternative methods (Montalbánlópez et al. 2012; Hegemann et al. 2015; Rutledge and Challis 2015).

Class II: Like lantibiotics, the second class of bacteriocins are synthesized as an inactive prepeptide that usually contain a typical double-glycine proteolytic processing position (Ennahar et al. 2000; Drider et al. 2006). However, some type II bacteriocins are typically Sec-dependent N-terminal signal sequence synthesized and secreted by the general secretory pathway.

Unlike lantibiotics, Class II bacteriocins do not experience extensive posttranslational modifications. Specific enzymes cleave off the leader peptide concomitant when the prepeptide completed translation, and it's translocated to the extracellular space by a specific ABC transporter that occasionally requires an auxiliary protein (Drider et al. 2006; Ennahar et al. 2000). However, there are a rising number of newly reported bacteriocins that lack leader sequences; these are of interest as they take effect directly after translation (Cintas et al. 2000; Fujita et al. 2007; Masuda et al. 2012).

L. lactis QU 5 and *L. lactis QU 14* can, respectively, produce lacticin Q and its homologue lacticin Z, which, respectively, correspond to the biosynthetic gene clusters lnqBCDEF and lnzBCDEF that are related to bacteriocins' secretion and self-immunity. In *L. lactis NZ9000*, overexpression of lnqQ resulted in the intracellular accumulation of lacticin Q (Iwatani et al. 2012). Through further analysis of the function of the nqBCDEF gene product, it was found that this gene cluster strictly

controls the secretion of lacticin Q into the extracellular space, whereas the control of the self-immunity system is relatively weak (Perez et al. 2014). The ABC transporter-type immunity LnqEF is sufficient to confer minimum immunity, whereas LnqBCD are thought to be accessory proteins that provide activity for LnqEF (Iwatani et al. 2013).

Class III: Both structural and immunity genes of megacins A-216 and A-19213 are plasmid encoded. Megacin A-216 contains 293 amino acid residues and shows a native molecular weight of 66 kDa (Kiss et al. 2008). The biologically active portion includes three parts called g, a, and b chains (32,855, 21,018, and 11,855 Da), corresponding to the full-length protein and two decomposition products, respectively (Kiss et al. 2008).

The genetic determinant of A-216 is encoded by the 5494-bp plasmid region. This plasmid region also contains the structural gene of A-216, which controls a 293 amino acid protein with sequence similarity to proteins with phospholipase A2 activity. The ORF gene near the megA gene encodes a 91 amino acid protein responsible for immunity of producer strain against megA-216. At least two other genes, including ORF 73 and a gene encoding a 188 amino acid protein, are required for the induction of megacin A-216 expression. Sequences encoding the 188 amino acid protein are highly similar to the RNA polymerase σ factor (Abriouel et al. 2011).

Class IV: AS-48 is a typical kind of Class IV bacteriocin. The AS-48-related gene clusters consist of ten major genes, including the structural gene AS-48A; AS-48B relates to a putative cyclase, AS-48C for a DUF95 protein related to immunity and production (Mu et al. 2014), AS-48C1D represents putative ABC transporter associated production, AS-48D1 is associated with typical immune protein production, and AS-48EFGH represents production of immune-related additional ABC transporters (Maqueda et al. 2008).The expression of AS-48 requires the expression of a large transcript that involves AS-48ABC which is posttranscriptionally processed, a second transcript including AS-48C1DD1EFGH, and a third transcript with a weak promoter that transcribes AS-48D1EFGH (Sánchezhidalgo et al. 2011a; Cebrián et al. 2014).

3.2.3 The Applications of Bacteriocins Derived from LAB

An overview of the applications of LAB and their bacteriocins is shown in Fig. 3.2, emphasizing the role of LAB bacteriocins in medicines and other industries.

1. The Application in Food

As for food, it's not enough to just consider about food's edibility, which means the shelf life. There are other things that are more important we need to pay attention to, such as nutrition, organoleptic, and, most importantly, consumer acceptability. The currently existing preservatives are not entirely satisfactory to the consumers. The foods which are alleged to be "free of additive" are often more popular to

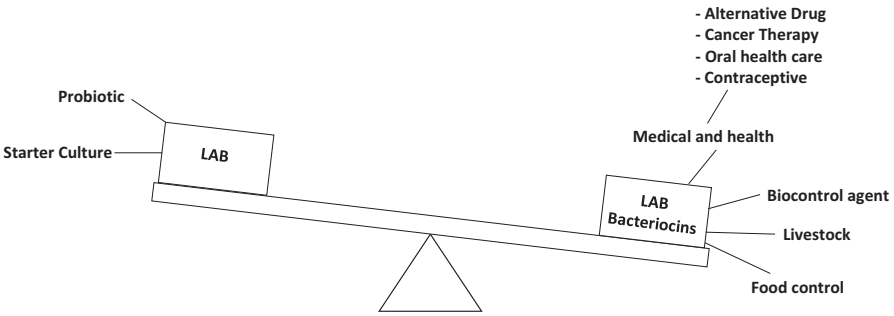

Fig. 3.2 The applications of LAB and their bacteriocins. (Liong 2015)

consumers. Bacteriocins as biopreservatives are more natural than chemosynthetic preservatives. Lactic acid bacteria bacteriocin is a kind of polypeptide, which can be digested by proteases secreted by digestive tract in the process of the body's digestion and will not result in any residues. On the other hand, bacteriocin can inhibit most corruption bacteria and foodborne pathogenic bacteria. In addition, bacteriocins are nontoxic, odorless, and colorless, which makes them likely to be an ideal protectant. The most widely used bacteriocin is lactic *Streptococci* bacteriocin. Nisin has been widely used in food production, and it can effectively inhibit the growth of harmful bacteria, improve the processing quality of the food, and prolong food storage time. Espitia et al. evaluated physical–mechanical and antimicrobial properties of nanocomposite film with pediocin and ZnO nanoparticles against *Staphylococcus aureus* and *Listeria* 7 monocytogenes (Espitia et al. 2013). Gassericin A, as food preservative from *Lactobacillus gasseri* LA39, was described as stable at 4 °C for 3 months, at 37 °C for 2 months, at 60 °C for 5 h, and at 100 °C for 30 min (Ahmad et al. 2017).

As for LAB bacteriocins, the bacteriocins from food-grade lactic acid bacteria (LAB) were qualified as an ideal food biopreservative predominantly because:

(i) It has been proven nontoxic to humans.
(ii) Does not alter the nutrients contained in the food.
(iii) Effective at low concentration.
(iv) It is still active under refrigeration conditions (Ahmad et al. 2017).

The preservative effect of bacteriocins can be used in the following aspects.

(1) Meat are usually perishable and susceptible to microbial contamination. Nisin can effectively control the growth of *Clostridium botulinum*, and nisin is acidic, so it can reduce the pH of the surrounding medium to reduce the residue of nitrite content and the formation of nitrosamines.
(2) In ancient times, LAB has begun to be applied to the fermentation food, which was mainly related to the beneficial effect of LAB on nutrition, color, flavor, and shelf life (Richard et al. 2006). Nisin which is added 30–50 mg/kg in dairy products can extend the shelf life and usually doubles the products' shelf life

under 35 °C. Adding 80~100 mg/kg nisin in canned condensed milk can reduce the time of sterilization by 10 min. 20 mg/kg of nisin added in UHT milk can completely inhibit the growth of spore bacteria in sterilized milk (Perez et al. 2014). Yogurt post-acidification process could be delayed for 3 days if 40 IU/mL of nisin is added, and the number of living bacterium stays above 107/mL, and the yogurt sensory quality is good. Nisin is the earliest preservatives used in the cheese. Using the mixture of *Streptococcus* acid-resistant bacteria and nisin as cheese starter can make cheese merit factor above 90% compared to the conventional method which is only 41% (Maisnier-Patin et al. 1992).

Until now, only nisin and pediocin PA-1 have been commercialized as food additives. However, other LAB bacteriocins like enterocin AS-48 (Sánchezhidalgo et al. 2011b) and lacticin 3147 (Suda et al. 2012) also showed promising perspectives to be used as biopreservatives in food. AS-48 is stable and soluble at wide range of pH and temperatures. This feature makes them able to be widely used in the food industry (Sánchezhidalgo et al. 2011b).

2. The Application in Medical Treatment

In the past decade, with the frequent occurrence of clinical drug resistance in the world, peoples' concerns to multidrug resistance have increased (Carlet et al. 2014). Particularly in Third World countries, most of the effective drugs have now turn out to be almost useless against a large number of the organisms. Microorganisms that are involved in serious infections have developed resistance to one or more than one common broad-spectrum antibiotics (Alanis 2005). The problem is caused not only by the microorganisms that resist effective antibiotics in different ways but also by the increase overprescribing and inadequate medications. Compared with other antibacterial compounds, bacteriocins are target-specific, broad-spectrum, and more effective antagonists. Bacteriocins can be used as alternative therapeutics against resistant pathogens, and there are several promising applications for controlling potential health risk factors (Papagianni 2003). The applications of bacteriocins in medical treatment can be summarized as follows:

(1) Skin and soft tissue infections are caused by *Staphylococcus aureus*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, *B. cereus*, *B. subtilis*, and *Listeria monocytogenes*. Nisin and some other bacteriocins have been described that they can effectively treat these skin diseases (Manosroi et al. 2010; Bowe et al. 2006; Kang et al. 2009; Izquierdo et al. 2009). For instance, the bacteriocin subpeptin JM4B produced by *B. subtilis* JM4 is very active against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp., and *Enterococcus faecalis* (Wu et al. 2005).
(2) The mouth is a necessary place which pathogens in food must pass. Thus some microorganisms will live in the oral cavity. Besides, oral cavity special environment easily leads to tooth decay, so oral rinse containing bacteriocins offers new possibilities for the development of oral rinse products. *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans* are regarded as the principal periodontal pathogens. Many bacteriocins, such as

subtilosin A against *Porphyromonas gingivalis* (MIC = 3.125–6.25 μg/mL) (Shelburne et al. 2007; Siegers and Entian 1995) and lacticin 3147, BLIS K12, and a bacteriocin of 114 kDa from *Lactobacillus paracasei* strain HL32 against *Porphyromonas gingivalis*, have been recorded to inhibit the growth of major oral pathogens (Hammami et al. 2011; Howell et al. 1993; Tagg 2004).

(3) The intestinal flora of humans and animals is closely related to LAB. Recent studies of LAB have demonstrated their efficacy in treating disorders of the intestinal flora. Lacticin L3147 has been shown to inhibit the growth of *Bacillus difficile* at low concentrations without affecting the normal human flora (Rea et al. 2007). This lantibiotic could be used as an effective oral therapeutic for overcoming *C. difficile*-induced diarrhea and gastric acid. *Helicobacter pylori* and antral gastritis are closely related to the occurrence of duodenal ulcer and gastric ulcer, and Nisin can inhibit *Helicobacter pylori*.

(4) In hospital settings, the main disease-causing pathogens are *Staphylococcus aureus*, pneumococci, enterococci, *Klebsiella pneumoniae*, *A. baumannii*, *Citrobacter freundii*, *E. coli*, and *Proteus* spp. (Ghodhbane et al. 2015; Michalet et al. 2007). Nisin and lacticin 3147 have significant inhibitory effect on various pathogens in the liver, kidneys, and spleen. But it's difficult to treat *Staphylococcus aureus*, enterococci, and pneumococci strains which are resistant to six or more antibiotics. Bacteriocins such as lacticin 3147 and nisin A have been found effective against MRSA and VRE (Piper et al. 2009).

Currently bacteriocin for the treatment of bacterial infection disease is usually an auxiliary treatment of bacterial intervention therapy trials; so far, no pure bacteriocin can be directly used as drugs in clinical therapy.

In addition to the above common application of the bacteriocins, bacteriocins can also be used for the following potential purposes. *G. vaginalis*, *Mycoplasma hominis*, *Prevotella bivia*, and *Mobiluncus curtisii* can lead to female vaginal diseases, but the antibiotics such as clindamycin and metronidazole we conventionally use can kill beneficial flora of the vagina. However, certain bacteriocins can help women against these bacteria, for example, bacteriocin lactocin 160 against *G. vaginalis* and subtilin A against *B. amyloliquefaciens* (Ahmad et al. 2017).

In addition to antimicrobial activity, bacteriocins also have the effect of killing spermatozoa. For example, fermenticin HV6b isolated from *Lactobacillus fermentum HV6b* (MTCC10770) shows strong function of sperm fixation and spermicidal activity. This characteristic makes it an attractive proposition for formulating antibacterial vaginosis and contraceptive products (Kaur et al. 2013).

Bacteriocins can not only make effects on human and animals, they can also be used in treating plant bacterial diseases. For example, ericin S is active against *C. michiganensis*, the causative agent of tomato bacterial canker. Purified ericin or its producer strain could be developed as a bioprotectant on tomato plants (Abriouel et al. 2011). In addition to the function of bacteriocins, Abriouel H puts forward that bacteriocins may have a potential to inhibit biofilm formation, thus can be used in pipeline cleaning technologies and consequently reduce biocorrosion, which may be beneficial for the environment.

In the future, we believe that as the more research unfolds, the application of LAB bacteriocins in health control will be more prosperous. Several bioengineered strategies will also be engaged to enhance the commercial potential of LAB and their metabolites in medical and health (O' Shea et al. 2013).

3.3 Exopolysaccharide (EPS)

Exopolysaccharides (EPSs) are long-chain polysaccharides composed of repeating sugar units in different ratios which mainly include glucose, galactose, rhamnose, and so on (De Vuyst and Degeest 1999a). Instead of permanently attaching to surface, these polysaccharides are secreted into their surrounding environments. This feature differentiates exopolysaccharides from capsular polysaccharides which always attach to the surface of the cell (Laws et al. 2001).

The most valuable application of EPSs from lactic acid bacteria (LAB) was the rheology and texture enhancement of yogurt products. Recently a higher demand for consumption of smooth and creamy products was evoked. It is usually catered by the increase of fat, sugars, and stabilizers. Despite of the cost, the consumers' demand for products with low fat or sugar and low levels of additives makes EPSs a viable alternative for yogurt products (Jolly et al. 2002). Although LAB EPSs have no own taste and flavor, the time the fermented milk product spends in the mouth was increased by the LAB EPSs, so an elevated perception of the human sense of taste is imparted (Duboc and Mollet 2001). In addition, EPSs will remain longer in the gastrointestinal tract (GIT), by which the colonization of probiotic bacteria is enhanced (German et al. 1999). Moreover, the LAB EPSs have been investigated to have the potential of antitumor effects Kitazawa et al. 1998), immunostimulatory activities (Hosono et al. 1997; Chabot et al. 2001; Shao et al. 2014), antihypertensive effects (Ai et al. 2008a, b), and functions to lower blood cholesterol (Nakajima et al. 1992a).

3.3.1 The Classification and Chemical Structure of EPS

For the EPSs from LABs, two categories can be summarized. The first category is homopolysaccharide which is composed of four different subgroups (i.e., α-D-glucans, β-D-glucans, fructans, and others). α-D-glucans or dextrans consist mostly of α-1,6-linked glucose monomers with various branched chains at position 3 but sometimes at positions 2 and 4 at a relatively lower probability, such as the EPSs from *Leuconostoc mesenteroides* subsp. *mesenteroides* and *dextranicum.* However, the EPSs form *Leuc. mesenteroides* (i.e., alternan), and *Streptococcus mutans* and *sobrinus* (i.e., mutans) are composed of both α-1,3- and α-1,6 linkages. β-D-Glucans consist of β-1,3-linked glucose monomers with β-1,2-branched chains, such as the EPSs from *Pediococcus* spp. and *Streptococcus* spp. Fructans consist mainly of

β-2,6-linked D-fructoses. For example, the *S. salivarius* EPSs (i.e., levan) have this structure and some β-2,1 side chains at the O_1 site. Similarly, other EPSs in the first category all consist of structurally identical repetitive elements with various glycosidic links, such as polygalactan.

The second category is heteropolysaccharides. This kind of EPSs are usually produced by LAB strains which have mesophilic and thermophilic life history, such as the mesophilic *Lactococcus lactis* subsp. *lactis* and *cremoris*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and thermophilic *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophiles*.

Interest has been evoked to the latter group of EPSs, for the reason that they play an important part in the mouthfeel of yogurt products. For example, as one of the crucial quality aspects, yogurt texture will become more creamy and smooth even though only tiny amounts of the EPSs are secreted. Moreover, LAB EPSs have its unique promising technical potential for novel fermented milk product development such as low-milk-solid, low-calorie, low-milk-fat, and creamier yogurt products. In addition, as nondigestible food fraction (Gibson and Roberfroid 1995), some polysaccharides may contribute to human health because of their antitumoral (Oda et al. 1983), antiulcer (Nagaoka et al. 1994), immunomodulating (Kitazawa et al. 1993), or cholesterol-lowering (Nakajima et al. 1992a) activity. Therefore, with both health and economic benefits, LAB EPSs have the potential for developing and exploiting functional food ingredients.

The chemical composition of LAB EPSs has long been controversial. (Sundman et al. (1953)) and (Nilsson and Nilsson (1958)) firstly investigated the nature of slime materials form LAB, which was found to be protein-like material. After this, however, some researchers speculated that glycoprotein or carbohydrate–protein complex are produced when milk fermentation would confer the ropy characteristics to yogurt (Macura and Townsley 1984; Garcia-Garibay and Marshall 2008; Toba et al. 1991), while other researchers isolated exo-polymer material which is enriched in carbohydrate material after further purification (Nakajima et al. 1990; Cerning et al. 1986, 1988,1992, 1994; Kojic et al. 1992; Norris et al. 1954; Wang et al. 1963; Bouzar et al. 1996; Cerning 1995). Finally, an agreement is achieved that the LAB exo-polymers are polysaccharides made up of repetitive elements and that many different categories are secreted Cerning 1995). However, the compositions of monosaccharide residues are very similar in appearance. Frequently D-galactose, D-glucose, and L-rhamnose are present but in different ratios (Ariga et al. 1992; Gamar et al. 1997; Doco et al. 1990; Nakajima et al. 1992b; Gruter et al. 1992, 1993; Yamamoto et al. 1994, 1995; Marshall and Cowie 1995; Robijn et al. 1995a, b, 1996a, b; Grobben et al. 1995, 1996, 1997; Mozzi et al. 1996; Stingele et al. 1996; Bubb et al. 1997; Lemoine et al. 1997; De Vuyst et al. 1998). Nevertheless, some special cases exist, for example, EPSs from *Lb. acidophilus* LMG 9433 (Robijn et al. 1996b), *Lb. helveticus* TYl-2 (Yamamoto et al. 1994) and NCDO 766 (Robijn et al. 1995b), and *Lb. rhamnosus* C83 (Gamar et al. 1997), *S. thermophilus* Sfi20 (Doco et al. 1990; Stingele et al. 1996), Sfi32 (Lemoine et al. 1997), LY03, BTC, and 480 (De Vuyst et al. 1998) lack rhamnose, EPSs from *Lb. paracasei* 34–1

contains only galactose (Robijn et al. 1996a), EPSs from *S. thermophilus* OR-901 contains only galactose and rhamnose (Ariga et al. 1992; Bubb et al. 1997), and EPSs from *Lb. sake* 0–1 only consists of glucose and rhamnose (Robijn et al. 1995a). Other residues, such as N-acetyl-aminosugars and *sn*-glycerol-3-phosphate, as well as the phosphate and acetyl groups could also be present (Nakajima et al. 1990, 1992b; Doco et al. 1990; Yamamoto et al. 1994, 1995; Marshall and Cowie 1995; Robijn et al. 1995a).

The different LAB EPS compositions found in the past may be to some extent because of the limits in isolation and purification techniques and consequently their poor performance, especially in the situation where complex media are selected, and the possibility that more than one kind of polysaccharides could be secreted by one strain. Grobben et al. once reported that different compositions of exopolysaccharides were characterized from the same *Lb. delbrueckii* subsp. *bulgaricus* strain, where different isolation and purification techniques were used under different fermentation conditions (Grobben et al. 1995, 1996, 1997). Marshall et al. isolated two different EPSs from the same *L. lactis* subsp. *cremoris* strain having different monosaccharide compositions and molecular masses (Marshall and Cowie 1995). Isolation of high- and low-molecular-mass EPS fractions was also performed form the same *Lb. delbrueckii* subsp. *bulgaricus* (Grobben et al. 1997) and *S. thermophilus* strain, but the monomeric composition of these EPSs does not differ. Ai et al. isolated the crude exopolysaccharides (LCP) from skim milk fermented by *Lb. casei* LC2W and fractionated these polysaccharides into three fractions (LCP1, LCP2, and LCP3) with the ratios of 35.74%, 12.61%, and 33.34% (w/w), respectively (Fig. 3.3) (Ai et al. 2008a, b).

LCP1 and LCP2 were composed of glucose, rhamnose, and galactose in molar ratios of 4.0:1.9:1.0 and 5.0:16.2:1.0, respectively. Besides these three sugars, LCP3 contained a given amount of mannose, the monosaccharide molar ratio of Glu:Rha:Gal:Man is 1.0:2.1:3.8:3.5 Ai et al. 2008a, b). Shao et al. separated two different kinds of EPS fractions from the *Lb. rhamnosus* KF5 in fermented skim milk by removing proteins, ethanol precipitation, anion exchange, and gel permeation chromatography. Fraction S1 was composed of glucose, arabinose, glucosamine, galactosamine, and galactose in an approximate molar ratio of

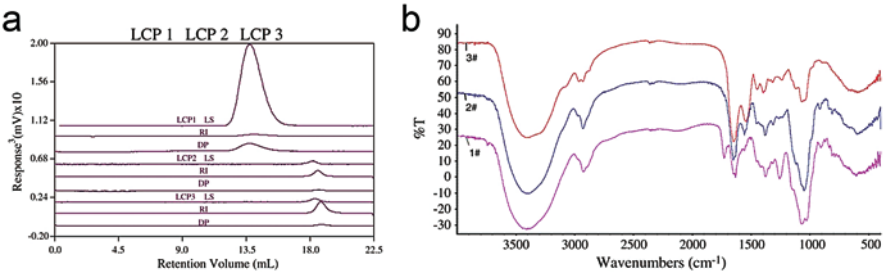

Fig. 3.3 (**a**) High-performance size exclusion chromatograms and (**b**) Fourier transform infrared spectra of EPS fractions LCP1, LCP2, and LCP3. (Ai et al. 2008a, b)

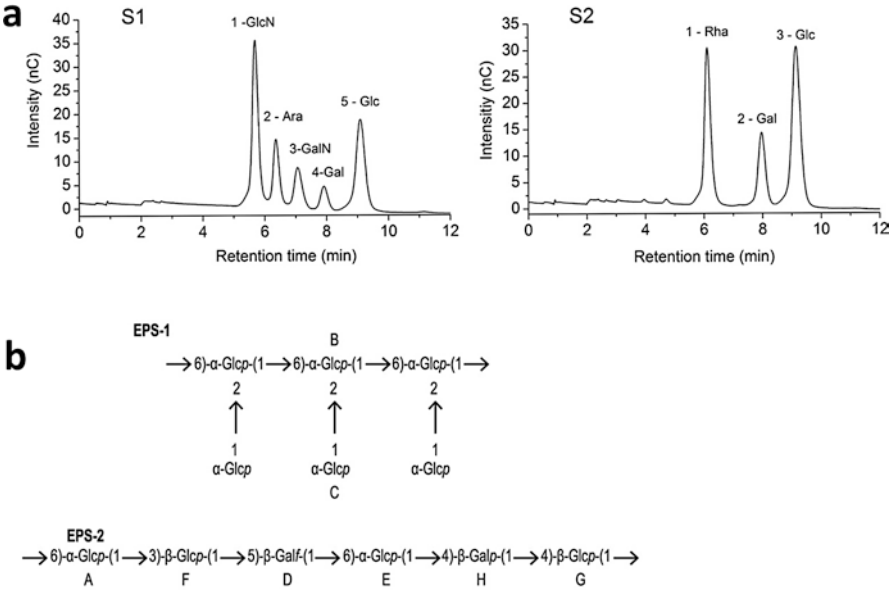

Fig. 3.4 (**a**) HPAEC profile of monosaccharide composition of EPS fractions (S1 and S2) from Lb. rhamnosus KF5 (Shao et al. 2014) and (**b**) structure of exopolysaccharides EPS-1 and EPS-2 from *Lb. johnsonii FI9785* (Dertli et al. 2013)

2.03:1.29:1.25:0.72:0.61, while fraction S2 contained rhamnose, glucose, and galactose in a molar ratio of approximately 1.73:1.47:1.00 (Fig. 3.4a) (Shao et al. 2014).

Dertli et al. isolated and purified exopolysaccharides from *Lactobacillus johnsonii* FI9785 which has previously been shown to act as a competitive exclusion agent to control *Clostridium perfringens* in poultry. Structural analysis by NMR spectroscopy revealed that *Lb. johnsonii* FI9785 can produce two types of exopolysaccharide, EPS-1 and EPS-2 (Dertli et al. 2013) (Fig. 3.4b).

LAB EPSs have molecular masses approximately ranging from 4.0×10^4 to 6.0×10^6 although EPS molecular mass is one of the factors affecting its processing characteristics (Cerning et al. 1992; Vandenberg et al. 1995). The three-dimensional conformation of a polysaccharide in solution is also closely correlated to its physical and rheological properties. In solution polysaccharide chains will experience some kind of topological rearrangements for the sake of transforming into a well-organized conformation in favor of intermolecular interactions and associations. So other factors, such as the intermolecular association abilities of polysaccharide molecules, could be important for a comprehensive understanding of the solution behavior. The secondary and tertiary conformation of EPSs is strongly dependent on the primary structure. Relatively small alteration in primary sequence might result in a tremendous effect on the three-dimensional conformation and corresponding processing characteristics of a EPS molecular mass. Doco et al. firstly determined the structure of the repeating unit of *S. thermophilus* heteropolysaccharide (Doco et al. 1990). Other structures of the repeating unit in branched LAB

EPSs have been confirmed recently via methylation, acetolysis, periodate oxidation, acid hydrolysis, enzymatic digestion, Smith degradation, 1D,2D ^{1}H-NMR spectroscopy techniques, etc. (Doco et al. 1990; Nakajima et al. 1992b; Gruter et al. 1992, 1993; Robijn et al. 1995a, b, 1996a, b; Stingele et al. 1996; Bubb et al. 1997; Lemoine et al. 1997; Dertli et al. 2013).

The size of repeating unit may range from a disaccharide to a heptasaccharide. Few common profiles are further shown to people, which introduce a question upon the relationship between EPS structures and the their texturizing properties. Exploring this relationship and exploiting the techniques modifying biopolymers that influence the properties of the native polysaccharides would be interesting. Specific enzymes can be applied to tailor the chemical structure of LAB EPSs and their functional properties. Integrating the biochemical and molecular biological researches on the LAB EPSs, future engineering of polysaccharide and perhaps oligosaccharide could be of great value given the explosive tendency of current functional food market.

3.3.2 The Biosynthesis and Genetics of EPS in LAB

The biosynthesis of a fraction of homopolysaccharides, such as dextrans, alternan, mutans, and levans, takes place outside the cell and thus requires specific substrates. For dextran and levan, highly specific glycosyl transferase enzymes dextran and levan sucrase are involved in polymerization reaction, where the energy required for polymerization is provided by hydrolysis of sucrose. Such kind of EPSs can be produced either using bacterial cells or cell-free systems (Cerning 1990).

On the other hand, the synthesis of heteropolysaccharides is in another way where polymerization of the repeating unit precursors takes place in the microbial cytoplasm (Cerning 1990, 1995), and several enzymes and proteins which are not necessarily unique to EPS formation are involved in the biosynthesis and secretion process. In the heteropolysaccharide biosynthesis process, polymerization and interconversions are both crucial steps, where interconversion further includes epimerization, decarboxylation, and dehydrogenation. Correspondingly, sugar activation and modification enzymes inevitably play an important part in this process because of their contributions in the sugar nucleotide (i.e., building blocks) formation.

For example, the UDP-glucose pyrophosphorylase was found to be correlated with EPS production in a ropy *S. thermophilus* strain but not in the non-ropy strain. Nevertheless, Escalante et al. found that it appears to have nothing to do with the EPS biosynthesis in any *S. thermophilus* strain examined (Escalante et al. 1998). Meanwhile, in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, the UDP-glucose pyrophosphorylase (responsible for the biosynthesis of UDP-glucose and UDP-galactose) was much more active in glucose-grown cultures than that in fructose-grown cultures. However, in the fructose-grown cultures, no enzyme activities were detected which led to dTDP-rhamnose biosynthesis (Grobben et al. 1996), and the

produced EPSs possessed a smaller proportion of galactose where the activity of UDP-galactose-4-epimerase was only faintly lower in these cells. So this enzyme doesn't cut within the sugar composition of the NCFB 2772 EPSs produced (Grobben et al. 1996). However, a correlationship between the activity of UDP-galactose 4-epimerase and EPS production was found in *L. lactis* (Forsén and Häivä 1981).

Glucose or the glucose element which comes from the hydrolysis reaction of lactose probably was the sugar source for the LAB heteropolysaccharide biosynthesis. It was also found that UDP-glucose pyrophosphorylase activities were high in these bacteria. Glucose-1-phosphate is likely a precursor for polysaccharide formation (Sjöberg and Hahnhägerdal 1989), where phosphoglucomutase is a hub point linking lactose degradation and EPS biosynthesis. If this branching point connects sugar catabolism and sugar anabolism, it will be interesting to engineer for overproduction of EPSs, of which the galactose element could be catabolized in glycolysis pathway, while the glucose element could be used for EPS synthesis. Therefore, the phosphoglucomutase downstream flux should be made sufficiently high (de Vos 1996). However, the experiment on the Gal^- (galactokinase) phenotype of *S. thermophilus* suggested that the function of UDP-galactose-4-epimerase and galactose 1-phosphate uridyltransferase is precursor biosynthesis for the EPSs.

Although the sugar nucleotide concentration in cell is a key factor on the monomeric composition of EPSs, the assembly of repetitive elements is also another important influencing factor. Hundreds to thousands of repetitive elements were assembled by sequential addition of carbohydrate elements via peculiar glycosyl transferases, coupled with the undecaprenyl phosphate carrier. For the first sugar residue, this isoprenoid glycosyl lipid carrier will act as recipient molecule. However, in Gram-negative LABs, only preliminary evidence for this kind of lipid carrier was found (Sutherland 1972, 1982, 1985, 1990). Moreover, the structural diversity of LAB EPSs implies that there must exist a vast set of glycosyl transferases involved in the assembly of the repetitive elements. However, these details have not been exploited thoroughly. Nevertheless, the heterologous expression of different combinations of glycosyl transferase genes will provide a new way of LAB polysaccharide engineering. Translocation of the assembled polysaccharide across the membrane to exterior and excretion in the environment or attachment to the cell are the last steps of EPS biosynthesis. Consequently, both the polymerization and transport processes will affect final amount of EPSs.

The nature and composition of EPSs are influenced by medium composition, growth phase, and generation time of bacterial growth, which are crucial factors in EPS biosynthesis and secretion. In order to increase exopolysaccharide production by *Lb. casei* LC2W, Ai et al. optimized the culture conditions (incubation temperature, incubation time, and inoculum concentration) which had significant influence on exopolysaccharide production (Ai et al. 2006).

The results showed that the optimal combination of the culture conditions for exopolysaccharide production was incubation temperature of 32.5 °C, incubation time of 26 h, and inoculum concentration of 4%. The maximum exopolysaccharide yield was 140 mg/L. Upon utilizing the optimal culture condition, the exopolysaccharide concentration obtained by experiment was 137 mg/L, with no statistical

difference with the predicted value at 5% level of significance. The fermentation by control of pH was further investigated to enhance the exopolysaccharide yield, and the results showed that the optimal pH for exopolysaccharide production was about pH 6.0 and the yield was increased by 15%, i.e., 160 mg/L (Ai et al. 2006). Yan et al. analyzed the relationship between EPS production and tolerance to artificial gastric and intestinal juices. The exopolysaccharide production of several *Bifidobacterium longum* strains isolated from infant and elder feces was determined, and the priming glycosyltransferase (pGT) gene fragments of these strains were amplified and sequenced. Results indicate that their tolerance correlated well with EPS production. The phylogenetic tree of the pGT gene sequence fragments showed that the pGT genes of infant-originated strains had better homology than those of elder-originated strains (Fig. 3.5) (Yan et al. 2017).

3.3.3 The Physiological Functionality of EPS

The probiotic functional food is now an expanding market. Probiotics are defined as live microbial food ingredients that are beneficial to health (Salminen et al. 1998). The food industry currently uses predominantly lactobacilli and bifidobacteria as

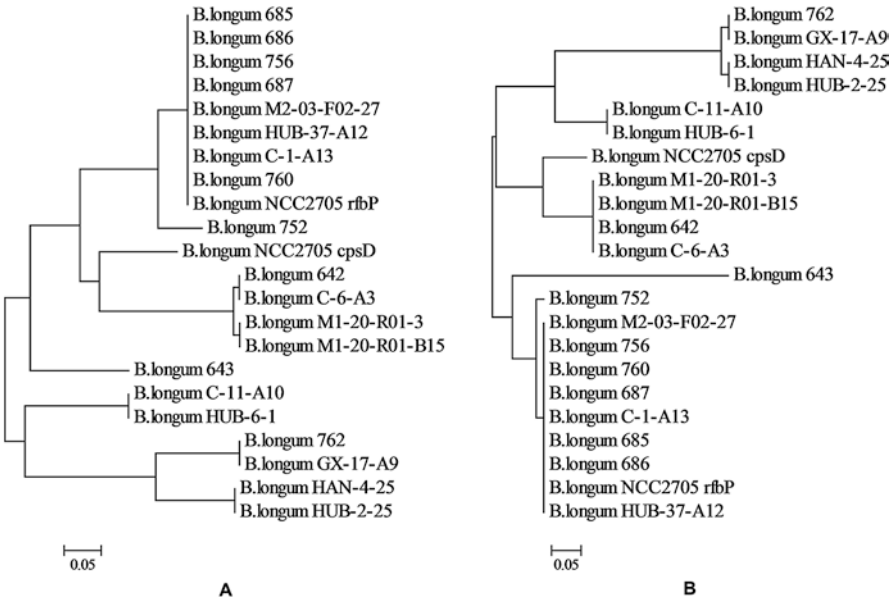

Fig. 3.5 Phylogenetic trees of the internal fragments of priming glycosyltransferases genes obtained by PCR amplifications (**A**) and the corresponding amino acid sequences (**B**)

probiotic bacteria, some of which produce EPSs. The health-promoting effect of EPS-secreting bacteria may have more or less relationship to the biological and physiological profiles of these secretory biopolymers. In these situations exopolysaccharides could be beneficial to human health in the way as employed by commercially available prebiotics (De Vuyst and Degeest 1999b).

The anticarcinogenic activity of LAB in fermented dairy products has been investigated. Kitazawa et al. reported that in mice if the lyophilized *Lactococcus lactis* subsp. *cremoris* KVS 20 was injected intraperitoneally, the growth of sarcoma-180 tumors would be suppressed. However, this strain did not emerge cytotoxicity to the sarcoma-180 tumor cells in vitro (Kitazawa et al. 1991). This implies that the antitumoral effect was mediated through the immune system where the slime material might be the principal factor in the antitumoral effect. In addition, a later study found that the slime material would largely induce an increase of the B-cell-dependent mitogenic activity (Kitazawa et al. 1992). Nakajima et al. found that the intraperitoneal administration of EPSs from *Lactococcus lactis* subsp. *cremoris* SBT 0495 in mice enhanced the production of specific antibodies (Nakajima et al. 1995). The starter commonly used in fermented milk products *Lb. delbrueckii* subsp. *bulgaricus* OLL 1073R-1, which produces EPSs, has been once reported to possess host-mediated antitumor activities (Kitazawa et al. 1998). The LAB EPSs were also reported to improve other immunological functions such as proliferation of T lymphocytes (Forsén et al. 1987), macrophage activation, and induction of cytokine production (Kitazawa et al. 1996). The water-soluble EPSs from kefir grains were reported to retard tumor growth when administrated orally, which is induced probably through T-cell and B-cell participation (Zubillaga et al. 2001). Dertli et al. assessed the effect of changes in cell surface characteristics on EPS production that may affect the ability of *Lb. johnsonii* colonizing the poultry host and exclude pathogens. Analysis of physicochemical cell surface characteristics reflected by zeta potential and adhesion to hexadecane showed that an increase in EPSs gave a less negative, more hydrophilic surface and reduced auto-aggregation (Fig. 3.6).

Auto-aggregation happened more frequently in mutants that have reduced EPSs, indicating that EPSs can mask surface structures responsible for cell–cell interactions. EPSs also affected biofilm formation, but here the quantity of EPSs produced was not the only determinant. A reduction in EPS production increased bacterial adhesion to chicken gut explants but made the bacteria more vulnerable to some stresses (Dertli et al. 2015). Further research is necessary to employ the EPSs or EPS-producing LAB into functional foods.

The susceptibility of EPSs to host digestion system might be a crucial point for its possible prebiotic characteristics. For the *Lactococcus lactis* subsp. *cremoris* NZ4010, its EPSs did not exert any protection role through the gastrointestinal transit of this bacterium (Looijesteijn et al. 2000). Also the resistance of EPSs to digestion was tested in vivo using rats. The rats were intervened by an EPS-rich diet for 2 weeks. Results showed that no EPSs were digested through the intestinal transit for the reason that the recovery of EPSs in the feces was nearly 100%. The resistance to biodegradability of this EPS was also studied by Ruijssenaars et al. who tested the biological breakdown susceptibility of several LAB EPSs

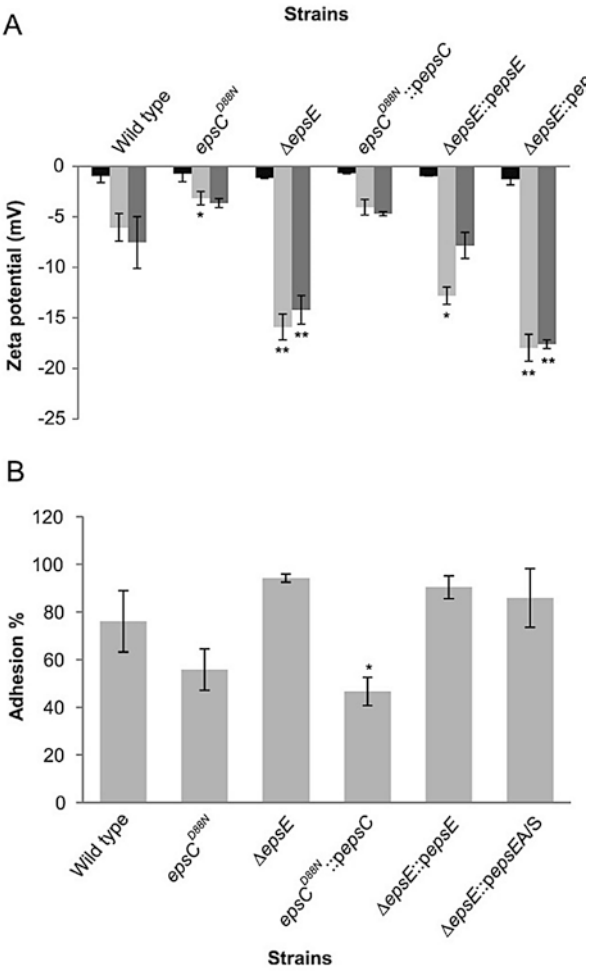

Fig. 3.6 Physiochemical characteristics of *L. johnsonii* FI9785 and mutant strains. (Dertli et al. 2015) (出版社*BMC Microbiol* 已成功索要,具体见邮件)

(Ruijssenaars et al. 2000). Gut microbiota could ferment *S. thermophilus* SFi39 and SFi12 EPSs, which is in contrary to EPSs from *Lactococcus lactis* spp. *cremoris* B40, *Lactobacillus sake* 0–1, *Streptococcus thermophilus* SFi20, and *Lactobacillus helveticus* Lh59. The resistance to biodegradability was related to the EPS primary sequences, where EPSs SFi39 and SFi12 had a single β-galactosyl element in the side chains, but EPSs B40 and 0–1 own two kinds of residues, one charged and the other uncharged, making these EPSs less accessible to hydrolases. Moreover, van Casteren et al. tested several hydrolases to B40 EPS, and no activity was found, except for the *Trichoderma viride* crude cellulase that showed activity to the galactosylphosphate residue (Casteren et al. 1998). More studies on microbial degradability, healthy effect to hosts, and the effects toward beneficial colonic bacteria proliferation are required.

3.3.4 The Technological Functionality of EPS

Firmness and water-holding power are the most important parameters of yogurt, and the structure of the gel might be related with these parameters. With the presence of increased EPSs, the cohesiveness and firmness of ropy strains fermented yogurts decreased (Hassan et al. 1996; Marshall and Rawson 1999). The casein micelles' association could be disturbed by EPSs, which will lead to a less firm coagulum. Studies on the microstructure of yogurt show that surrounding the EPS-producing microbes, there are some void inches which in turn would affect the matrix integrity. Yogurts made by ropy cultures had the highest ability to retain water and decrease the syneresis susceptibility (Hassan et al. 1996).

Though there was no very clear correlationship between the viscosity of yogurt and EPS concentration in it, in stirred yogurt the LAB EPSs' contribution to the rheological properties is a common proposed assumption (Hess et al. 1997; Rawson and Marshall 2003; Marle et al. 1999). When using the non-ropy LL yogurt culture, even though the polymer amount produced was nearly the same as that of ropy RR culture, a great difference in viscosity was found. In this situation the apparent viscosity of yogurt was profoundly influenced by the spatial structure of the protein network (Marle and Zoon 1995). Wacher-Rodarte et al. and Sebastiani et al. once reported that thermophilic LAB yogurts' viscosity values were not positively correlated with the amount of EPSs present (Wacher-Rodarte et al. 1993). Unless the production of a given type of EPSs is increased, the viscosity value also enhanced (Sebastiani and Zelger 1998). Through adding peptone to the milk, *S. thermophilus* could increase the EPS production, and then a concomitant increase in viscosity was observed.

In fact, the differences in viscosity improvement via different strains are principally a consequence of the differences in EPS intrinsic viscosity. Tuinier et al. described the physical traits of *Lactococcus lactis* subsp. *cremoris* B40 EPSs, of which the intrinsic viscosity and the concentration as well as shear rate could be predicted from the hydrodynamic radius and molar mass (Tuinier et al. 1999a). In the studies exploring the physical properties of aqueous solutions from *L. lactis* subsp. *cremoris* SBT 0495 EPSs, similar findings were also obtained, where the EPSs have the same repeating units as EPS B40 (Higashimura et al. 2000; Oba et al. 1999).

Normally speaking, the molar mass that is relatively high and the side chain that is relatively stiff are needed for the sake of getting a high yogurt viscosity. The effect of molar mass and radius of EPSs is also deduced by Faber et al. (1998). In the case of *Lactobacillus sakei* 0–1 EPSs, its average molar mass is at the same order of magnitude compared to that of xanthan gum, but its intrinsic viscosity is higher (Van den Berg et al. 1995). Monosaccharides connected by β(1–4) bonds produce stiffer chains compared with α(1–4) or β(1–3) bonds (Tuinier 1999). Meanwhile extent of branches and side chain groups play an important part in the EPSs' stiffness. In the case of *Lactobacillus helveticus* K16 EPSs, the special branching pattern of its molecular structure may be the reason why in aqueous solution these EPSs showed such high viscosity (Yang et al. 2000). In another case

if researchers terminally removed the galactosyl residues from *Lactococcus lactis* subsp. *cremoris* B39 and B891 EPSs, these polysaccharide molecules were apt to be given a decreased chain stiffness and thickening efficiency (Tuinier et al. 2015). On the other hand, if researchers removed off the acetyl group of B891 EPS, this modification did not affect its stiffness. In addition, depending on the solution ionic strength, the EPS intramolecular repulsion forces could be increased by the presence of charges, such as the negative charge of EPS phosphate group, which results in an enhancement of the hydrodynamic volume and thus the intrinsic viscosity. Several electron microscopy studies have been implemented (Skriver et al. 1995), and Tuinier et al. found that the depletion effects of EPSs with casein micelles happened in natural milk pH conditions (Tuinier et al. 1999b).

By means of metabolic engineering, biosynthesis of desired structure of EPSs could be accomplished. Some success has been achieved to overexpress the EPSs. The *epsD* gene was expressed under control of the NICE system. Compared to the wild-type *L. lactis* strain, the induction with nisin A results in a higher EPS production (van Kranenburg et al. 1999). Meanwhile, the attempts to produce new kind of EPSs have also been reported to be successful. When a non-EPS-producing strain was transferred into a gene cluster which encoded the EPSs from *S. thermophilus* SFi6, the strain would secrete another kind of EPS, unexpectedly, with a different structure, where the backbone N-acetylgalactosamine was substituted by galactose, and at position 6 of the glucose, the galactose side chain disappeared (Stingele et al. 1999). Horn et al. found that the *epsC* gene from the smooth mutant of *Lb. johnsonii* FI9785 had a single substitution (G–A) in the coding strand. Another gene in the cluster, *epsE,* plays a role in cell aggregation and a reduction in exopolysaccharide content (Horn et al. 2013).

However, due to low production levels, currently most LAB strains are not suitable for the commercially profitable production of EPSs. These kinds of microbes are more appropriate as functional additives, where the biopolymers are synthesized in situ, conferring natural fermented products with an improvement of rheological and prebiotic characteristics.

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Chapter 4
Metabolites of Lactic Acid Bacteria

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Contents

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W. Chen, A. Narbad, *Lactic Acid Bacteria in Foodborne Hazards Reduction*,
https://doi.org/10.1007/978-981-13-1559-6_4

Abstract Lactic acid fermentation is among the oldest forms of food preservation, but to extend the shelf life is only the start of which lactic acid bacteria (LAB) affects our foods. For example, lactic acid fermentation might develop specific textures, enrich some characteristic sensory properties, and even afford human health maintaining and promoting benefits. It has long been believed that LAB exerts those functions by their various metabolites. To this end, their potential use as cell factories for desired microbial metabolites is receiving extensive attention by the food and pharmaceutical industries. More importantly, the application of genetic engineering and metabolic engineering of LAB promotes the production of both the primary and the more complex secondary metabolisms. This chapter will summarize recent findings about metabolites (such as lactic acid, short-chain fatty acids, γ-amino butyric acid, conjugated linoleic acid, bacteriocin and bacteriocin-like, etc.) of LAB as well as their potential in industries.

Keywords Lactic acid bacteria (LAB) · Metabolites · Applications · Biochemical mechanism

4.1 Introduction

Lactic acid fermentation is among the oldest forms of food preservation, but to extend the shelf life is only the start of which lactic acid bacteria (LAB) affects our foods. For example, lactic acid fermentation might develop specific textures, enrich some characteristic sensory properties, and even afford human health maintaining and promoting benefits. It has long been believed that LAB exerts those functions by their various metabolites. To this end, their potential use as cell factories for desired microbial metabolites is receiving extensive attention by the food and pharmaceutical industries. More importantly, the application of genetic engineering and metabolic engineering of LAB promotes the production of both the primary and the more complex secondary metabolisms. This chapter will summarize recent findings about metabolites (such as lactic acid, short-chain fatty acids, γ-amino butyric acid, conjugated linoleic acid, bacteriocin and bacteriocin-like, etc.) of LAB as well as their potential in industries.

4.2 Aromatic Metabolites

Flavor is an important property of food products, and it majorly determines its acceptability and preference for customers. The formation of flavors of dairy fermentations, such as cheeses and yogurts, involves a series of (bio)chemical conversions of milk components by microorganisms in the starter cultures. There are three main metabolic pathways that have been identified: the metabolic process of carbohydrate (glycolysis), fat (lipolysis), and protein (proteolysis). The predominant microorganisms in these starter cultures of dairy fermentations are lactic acid bacteria (LAB), including *Lactococcus lactis*, *Lactobacillus* species, *Streptococcus thermophilus*, *Bifidobacterium* species, and *Leuconostoc* species (Smit et al. 2005).

4.2.1 The Formation of Flavor Compounds

4.2.1.1 Flavor Compounds Produced from Carbohydrate Fermentation by LAB

Lactose is the major energy substance and carbon source for LAB in fermented dairy products. There are two kinds of fermentation style for LAB, homofermentation and heterofermentation. They generate different metabolites of lactose fermentation; homofermentation majorly generates lactic acid, whereas heterofermentation forms some other metabolites, including ethanol, carbon dioxide, and acetic acid. Interestingly, under certain conditions, such as carbon limitation or aerobic culture, a homofermentation can be changed into a mixed-acid metabolism which produces varied metabolites, including several aroma compounds, such as acetaldehyde and diacetyl (Chen et al. 2017).

4.2.1.2 Flavor Compounds from Protein Fermentation by LAB

The proteolysis of caseins plays an important role in flavor formation in dairy fermentation, which contains two steps: proteolysis and amino acid metabolism. Firstly, casein is hydrolyzed to oligopeptides by cell-envelope proteinases (CEPs), furtherly degraded to amino acids by peptidases. Next, free amino acids can be metabolized to various flavor components, such as ammonia, amines, aldehydes, phenols, indole, alcohols, and so on. Particularly, branched-chain amino acids (e.g., valine, leucine, isoleucine), aromatic amino acids (e.g., phenylalanine, tyrosine, tryptophan), and sulfuric amino acids (e.g., cysteine, methionine) are the major precursors for flavor formation (Liu et al. 2010). Although these sulfur compounds are important for cheese flavors, some of them are off-flavors for yogurt.

4.2.1.3 Flavor Compounds from Lipids in LAB

The lipolysis of fat is also crucial to form aroma compounds in dairy fermentation. It has been reported that the lipolysis plays an important role in the flavor development of long-ripened cheeses, while it exerts a limited role in yogurt.

Firstly, lipid is degraded to fatty acids (FAs) by lipase. Unsaturated FAs are oxidized to form hydroperoxides which rapidly convert to hexanal or unsaturated aldehydes (Cheng 2010). Unsaturated FAs also can convert to four or five hydroxy acids to form cyclic compounds. The predominant cyclic compounds in dairy products are γ- and δ-lactones which provide strongly fruity flavors. Whereas, some lipid oxidation and lipolysis can also result in off-flavor. For example, aldehydes and ketones produced from lipid oxidation cause the stale and "oxidized" flavors to dairy products. Furthermore, LAB contains lots of esterases which can directly synthesize flavor esters from glycerides and alcohols via an alcoholysis reaction (Holland et al. 2005). For example, LAB can esterify ethanol with butyric acid and hexanoic acid to form ethyl butanoate and ethyl hexanoate. Interestingly, diglycerides and monoglycerides are preferred substrates, rather than triglyceride, to form flavor esters (Medina et al. 2004).

4.2.2 Representative Flavor Compounds

4.2.2.1 Acetaldehyde

Acetaldehyde, a colorless liquid with the chemical formula CH_3CHO, is another important aroma compound in dairy products, which exhibits a green apple or nutty flavor.

Acetaldehyde is formed by both *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurts. Acetaldehyde is a key aroma compound for good flavor in yogurt. Good flavored yogurt requires proper levels of acetaldehyde in a range of 23–40 mg/kg (Cheng 2010). The major metabolic precursor for acetaldehyde biosynthesis is pyruvate, which converts to acetaldehyde by α-carboxylase or aldehyde dehydrogenase (Chen et al. 2017).

4.2.2.2 Butanedione

Butanedione, also named as diacetyl, is a yellow or green liquid with the chemical formula $(CH_3CO)_2$ which might provide buttery flavor to various fermented milk products, such as cheese, cream, yogurt, and so on. In addition, in the food industry, diacetyl is also used as flavor agent in non-milk products, such as soft drinks, cold drinks, baked goods, candy, and other products. However, diacetyl is an important off-flavor source of beer and wine.

Production of Diacetyl

In nature, diacetyl is a by-product of lactic acid fermentation especially by *Lactococcus lactis* and/or *Leuconostoc* spp. from citrate metabolism. Citrate metabolism could provide pyruvate and lead to the accumulation of α-acetolactate which is the precursor to form diacetyl through an oxidative decarboxylation reaction. Fresh milk contains 0.2% citric acid which might be easily converted to pyruvic acid without involvement of nicotinamide adenine dinucleotide (NAD).

Strategies of Promoting Diacetyl Production

Considering it has a great value as aroma compound, high production of diacetyl has attracted a lot of attention now.

(a) *Optimization of Culture Conditions*

In the fermentation with *Lactococcus lactis*, the production of diacetyl is influenced by various extracellular factors, such as culture medium, the type and percentage of carbon and nitrogen source, growth temperature, pH, oxygen, and so on. Studies have found that the production of diacetyl by *Lactococcus lactis* cultured in aerobic conditions was far higher than its production in anaerobic conditions. Under aerobic conditions, adding citric acid to the medium can improve the output of diacetyl. When reaching the acidity and the flavor, cooling the products immediately could efficiently inhibit the activity of diacetyl reductase to save diacetyl.

(b) *Genetic Engineering and Metabolic Engineering*

The production of diacetyl is closely related to several key enzymes in the process of fermentation, including citrate lyase, oxaloacetic acid decarboxylase, alpha acetyl synthetase, NADH oxidase, alpha acetyl lactic acid decarboxylase, diacetyl reductase, and so on. Using molecular biology techniques to make these enzymes, inactivation or excess expression can increase the intermediate accumulation, including pyruvic acid and alpha acetyl lactic acid, to change the diacetyl metabolic pathways and eventually increase the yield of diacetyl.

4.2.2.3 Esters

Esters are a large group of volatile compounds which are usually present in various fermented foods at concentrations above the sensory threshold. Frequently, they are ethyl esters of the straight-chain fatty acids of C2–C10 and contribute to the development of the characteristic “fruity” type flavors in cheeses. During cheese processing, lipolysis might result in products of a variety of free fatty acids (FFA) which directly affect the flavor. In some way, esters may mask the impact of off-flavors induced by the high levels of short-chain FFA. It is well-documented that lactic acid bacteria such as *lactobacilli*, *lactococci*, *enterococci*, and *streptococci* contributed to the flavor ester compound formation via the esterase/lipase systems, but the underlying mechanism remains largely unknown. Therefore, further study in these areas is also greatly required.

4.3 Organic Acid

4.3.1 Lactic Acid

Lactic acid is a three-carbon α-hydroxycarboxylic acid with a single chiral center, which therefore exists in two stereoisomers, D- and L-lactic acid (Fig. 4.1).

4.3.1.1 Lactic Acid Formation

It is well-known that lactic acid formation by lactic acid bacteria (LAB) is classified as two types, homofermentative and heterofermentative pathway.

(a) *Homofermentation*

This process contains two steps (Fig. 4.2). Firstly, called glycolysis or Embden–Meyerhof–Parnas (EMP) pathway, glucose is converted to pyruvic acid. Later, pyruvic acid is reduced to lactic acid. Thus, lactic acid is the sole product from homofermentative pathway, which could lead to a maximum theoretical yield of 2 mol lactate per mol glucose:

$$\text{Glucose} + 2\text{P} + 2\text{ADP} \rightarrow 2\text{Lactate} + 2\text{ATP} + 2\text{H}_2\text{O} \tag{4.1}$$

Microorganisms that use only this glucose metabolism route include *Lactobacillus acidophilus*, *Lactobacillus amylophilus*, *L. bulgaricus*, *Lactobacillus helveticus*, and *L. salivarius* (Martinez et al. 2013).

(b) *Heterofermentation*

In contrast, a heterofermentative route of glucose is metabolized through the phosphoketolase (PK) pathway. This process also forms some coproducts such as CO_2, ethanol, and/or acetic acid along with lactic acid (Fig. 4.3). In this pathway, it results in a maximum theoretical yield of 1 mol lactate per mol glucose.

$$\begin{aligned}\text{Glucose} + 2\text{NAD}(\text{P}) + \text{Pi} + 2\text{ADP} &\rightarrow \text{Lactate} + \text{Acetate} \\ +\text{CO}_2 + 2\text{NAD}(\text{P})\text{H} + 2\text{ATP} + \text{H}_2\text{O}&\end{aligned} \tag{4.2}$$

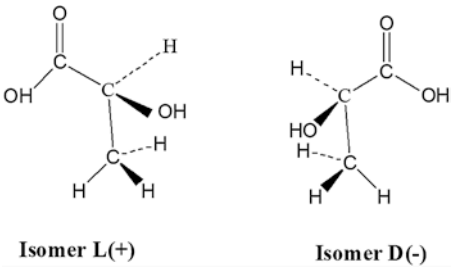

Fig. 4.1 Structure of D (−) and L (−) isomers of the lactic acid (Martinez et al. 2013). Reprinted from Martinez et al., Copyright 2013, with permission from Elsevier

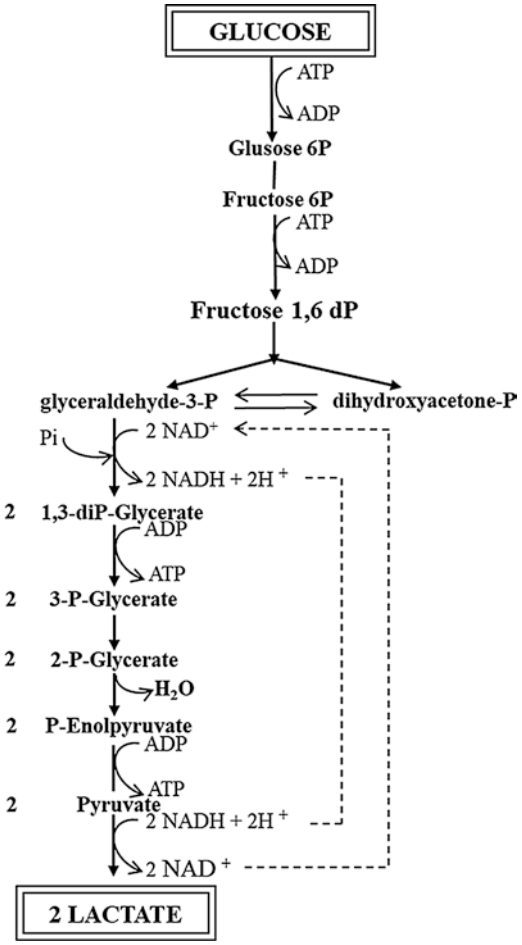

Fig. 4.2 The homofermentative pathway of glucose in LAB (Martinez et al. 2013). Reprinted from Martinez et al., Copyright 2013, with permission from Elsevier

Microorganisms that use only this glucose metabolism route include *Lactobacillus brevis*, *L. fermentum*, *L. parabuchneri*, and *L. reuteri* (Martinez et al. 2013).

4.3.1.2 Applications of Lactic Acid

Lactic acid (LA) is one of the most important products of LAB, which attracts a lot of interest in its wide applications, mainly in food, pharmaceutical, cosmetic, and chemical industries.

Food industry is the most important area where lactic acid is used, such as in the production of yogurt and cheese. In the production of yogurts, lactic acid is mainly generated by the co-fermentation of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. In the production of cheese, lactic acid promotes the aggregation of casein micelles by declining the pH in cheese. In addition, lactic acid and its salts are widely used as food additives, which are considered generally recognized as

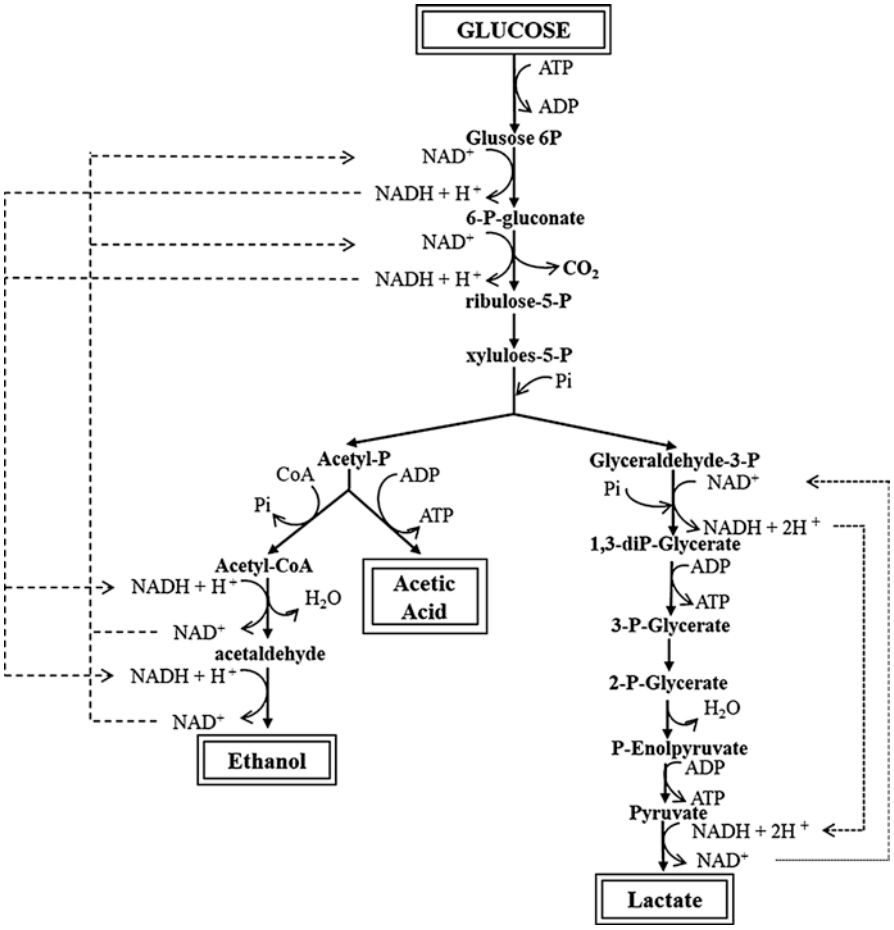

Fig. 4.3 The heterofermentative pathway of glucose in LAB (Martinez et al. 2013). Reprinted from Martinez et al., Copyright 2013, with permission from Elsevier

safe (GRAS) by the Food and Drug Administration (FDA) (Martinez et al. 2013). Therefore, lactic acid can be directly added in some food product, on the one hand can adjust flavor to form the desired sensory characteristics, and on the other hand can prevent the proliferation of undesirable microorganisms. In addition, in the process of grain production, lactic acid also is formed spontaneously, which can result in changes in the flavor of final products and avoidance of the proliferation of pathogenic bacteria (Lee and Lee 1993).

Additionally, lactic acid is also an important raw material for producing small or large compounds including propylene glycol and acrylic polymers (Sanmartin et al. 1992). Their polymers possess well biodegradable and biocompatible properties, which have potential in manufacturing industrial materials for packaging, labeling,

and prosthetic devices. For example, the polylactic acid is widely used in the textile and pharmaceutical industries (Singhvi et al. 2010).

4.3.2 Acetic Acid

Acetic acid is one of the most important products of LAB, which comes from heterofermentative pathway of glucose in LAB, such as *L. brevis*, *L. fermentum*, and *Bifidobacterium*. Acetic acid possesses strong bacteriostatic activity and wide range of bacteriostasis. Acetic acid can inhibit the growth and development of yeast, fungi, bacteria, and other harmful microorganisms. Acetic acid and the other organic acids produced by LAB can reduce the pH value, destroy the bacterial membrane of Gram-negative bacteria, and interfere with the formation of bacterial enzymes, thus exerting the bacteriostatic effect. Therefore, acetic acid and the other organic acids produced by LAB are widely utilized in food preservation.

Additionally, acetic acid is also beneficial to human health. Acetate is the most abundant short-chain fatty acids in the periphery (Cummings et al. 1987). Increasing evidence strongly suggested that acetate might play an important role in maintaining host physiology homeostasis through G protein-coupled receptors (GPCRs), especially GPR43/FFAR2 (Koh et al. 2016). The healthful effects of acetate are shown as follows:

4.3.2.1 Host Metabolism

A growing number of research have indicated that acetate promoted weight loss and improved glycemic control (Yamashita et al. 2007; Gao et al. 2009). In rodent and human experiments, supplementation with acetate could effectively improve glucose tolerance. The underlying mechanism is that acetate activating GPR43 results in the secretion of enteroendocrine hormones, such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) levels, to affect satiety and intestinal transit. Moreover, acetate activating GPR43 also leads to the promotion of intestinal gluconeogenesis (IGN) effect (De Vadder et al. 2014). Additionally, supplementation with acetate also could reduce obesity by activating GPR43 in white adipose tissue (WAT), which is beneficial to adipose metabolism (Kimura et al. 2013).

4.3.2.2 Gut Health

Acetic acid is beneficial to the large intestinal peristalsis to improve constipation. Acetic acid also could inhibit the proliferation of Ehec O157 in the intestinal tract and the infection of influenza virus (Yasui et al. 2004; Tripolt et al. 2013; Akoglu et al. 2015).

4.3.2.3 Nervous System

Interestingly, acetic acid plays various roles on the host brain. For example, a small amount of acetate could cross the blood–brain barrier (BBB) and then activate hypothalamic neurons to induce satiety (Frost et al. 2014). Recently, it is reported that acetate is crucial to microglia maturation in the brain via activating GPR43. Furthermore, acetate could modulate the permeability of the BBB (Koh et al. 2016).

4.3.3 Gamma-Aminobutyric Acid (GABA)

Gamma-aminobutyric acid (GABA) is a non-protein amino acid, and it is a well-known major inhibitory neurotransmitter in the central nervous system. GABA is widespread in mammals and plants; however, its level is low by nature. Therefore, GABA is largely produced by chemical synthesis or bioconversion by using microorganisms (Rizzello et al. 2008; Zhao and Shah 2014). Whereas, chemically synthesized GABA is prohibited in the food industry; therefore, the bioconversion of GABA by using food-grade microorganisms, especially LAB, will be an important method to produce GABA or GABA-rich foods (Lee and Paik 2017).

Currently, a large amount of LAB species/subspecies isolated from various environments have been confirmed to produce GABA. It shows that there is a vast difference in GABA production capacity of different LAB. It has been reported that acid-based fermented foods possess the high GABA producers, such as Korean kimchi and pickled vegetables. It is well-known that GABA is produced by glutamate decarboxylase (GAD) which catalyzes the decarboxylation of L-glutamate to form GABA. The LAB with high GABA contents could eliminate intracellular protons during the GAD catalytic process, which is beneficial to maintain the intracellular pH homeostasis under acidic conditions (Hutkins and Nannen 1993). In addition, it has been reported that most of LAB with high productivity of GABA belong to *Lb. brevis* and *Lb. plantarum*.

Amounting of evidence has revealed that GABA possessed of a lot of physiological functions including antidepressant, antianxiety, and antihypertensive effects, and so on (Yoshimura et al. 2010; Ogunleye et al. 2015). It has been reported that GABA or GABA-rich foods produced by LAB also exerted the antidepressant and antihypertensive effects on host (Wu and Shah 2017).

4.3.4 Conjugated Linoleic Acid

Conjugated linoleic acids (CLAs) are a group of positional and geometric isomers of linoleic acid (LA) with conjugated double bonds, which are majorly existed in milk and meat products (Steinhart et al. 2003).

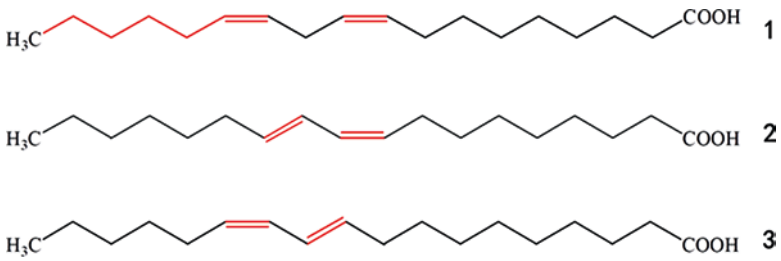

Fig. 4.4 Structure of linoleic acid and its major CLA derivatives (Benjamin and Spener 2009). Note: *1*. Linoleic acid; *2*. cis-9, trans-11-CLA (9-CLA); *3*. trans-10, cis-12-CLA (10-CLA). Reprinted from Benjamin and Spener (Open Access), Copyright 2009, with permission from BioMed Central (BMC)

Cis-9, trans-11-CLA (9-CLA) and trans-10, cis-12-CLA (10-CLA) are the predominant isomers of CLA, which are primarily derived from linoleic acid (Fig. 4.4). Accordingly, most of the researches focus on evaluating the biological effects of these major CLA isomers (Steinhart et al. 2003).

In comparison with the natural conversion, CLAs produced via chemical isomerization of LA appear to be complex mixtures of isomers. Therefore, microbial synthesis of CLA may be a promising method for industrial production (Khosravi et al. 2015).

Many bacteria have been reported to convert free LA into CLA, but some strains of lactic acid bacteria (LAB) have attracted more attention than other CLA-producing strains due to their health-promoting effects, such as *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* (Kuhl and De Dea Lindner 2016).

The Health Benefits of CLA

CLA has received much attention in recent years because of its health-promoting benefits including anti-obesity, anti-carcinogenesis, anti-inflammation, and anti-diabetes activities.

(a) *Anti-obesity*

Major biochemical mechanisms of CLA related to its anti-obesity capacity are summarized in Table 4.1 (Benjamin and Spener 2009).

Amounting of researches have suggested that there is a multiple mechanism for the anti-obesity capacity of CLA. Primarily, CLA could bind to PPARγ to control the differentiation of preadipocyte. Moreover, CLA could decrease energy or food intake, increase energy expenditure, decrease preadipocyte differentiation and proliferation, decrease lipogenesis, and increase lipolysis and fat oxidation (Salas-Salvado et al. 2006). Furthermore, CLA also could exert its anti-obesity effect by decreasing leptin levels, increasing adipocyte apoptosis, and increasing tumor necrosis factor-α (TNFα) levels (Benjamin and Spener 2009).

Table 4.1 Major biochemical mechanisms of the anti-obesity effect of CLA

Biochemical action	Experimental evidence
Preadipocyte proliferation	Inhibited proliferation
Preadipocyte differentiation	Human preadipocytes do not differentiate in the absence of a PPARγ ligand like CLA
Fatty acid oxidation	Carnitine palmitoyltransferase activity increased by dietary CLA
Adipose tissue lipid synthesis	Inhibition of de novo lipogenesis through downregulation of acetyl-CoA carboxylase and fatty acid synthase
Lipolysis	Increased lipolysis and decreased fat
Energy expenditure	Increased oxygen consumption and energy expenditure by 10-CLA
Stearoyl-CoA desaturase	Inhibition at protein or activity level, by posttranslational modification
Plasma leptin	Decrease in serum leptin, a hormone regulating fat level
Apoptosis	Induce apoptosis in adipocytes
Tumor necrosis factor-α	Increased expression of TNFα and low fat

Benjamin and Spener (2009)

(b) *Anti-carcinogenesis*

In mice and rats, evidence has indicated that CLA exerted potent inhibitory effects on skin, stomach, mammary, or colon tumors (Kritchevsky 2000). Additionally, in vitro studies have shown that CLA could prevent the proliferation of murine myeloid leukemia (WEHI-3B JCS), human colorectal (HT-29, MIP-101), and prostate (PC-3) colorectal cells (Palombo et al. 2002; Lui et al. 2005). Interestingly, in vivo human studies also verify that CLA possesses potent antiproliferative effects on breast and prostate cancers (Palombo et al. 2002; Ochoa et al. 2004).

It is suggested that the anti-carcinogenesis effects of CLA might be attributed to blocking the growth and metastatic spread of tumors. CLA is fast acting to suppress both malignant and benign tumors (Belury et al. 2002). It seems that 10-CLA isomer works preferentially through modulating apoptosis and cell cycle, while 9-CLA isomer works primarily through affecting arachidonic acid metabolism (Ochoa et al. 2004). The molecular mechanisms underlying the antiproliferative effects of CLA are complex. However, it may be partly through reducing cell proliferation, lipid oxidation, vitamin A, and prostaglandin (PG) metabolisms of tumors (Cheng et al. 2003; Chujo et al. 2003; Salas-Salvado et al. 2006; Gorocica-Buenfil et al. 2007).

(c) *Anti-diabetes*

A large number of evidence have supported that supplementing the diet with CLA could effectively inhibit diabetics, especially type II diabetics. Amounts of research suggest that the 10-CLA is the most effective CLA isomer to inhibit type II diabetes (Belury et al. 2003).

The molecular mechanisms underlying the anti-diabetes effects of CLA can be concluded in several aspects. Firstly, CLA could primarily bind with transcription factors, such as PPARγ, to affect glucose metabolism (Hammarstedt et al. 2003). Moreover, CLA could regulate the expression of genes (like uncoupling proteins) related to glucose and lipid metabolism (Ryder et al. 2001). Another possible mechanism is via the sensitization of the adiponectin by CLA and then to enhance insulin sensitivity (Nagao et al. 2003).

(d) *Immunomodulation*

In vitro and vivo studies have demonstrated that CLA could effectively modulate immune function.

Reports demonstrate that all the active CLA isomers, 9- and 10-CLAs, can elicit both the innate and adaptive immune responses to regulate immunity, such as to control the production of eicosanoids, cytokines, and immunoglobulin (Albers et al. 2003; O'Shea et al. 2004). For example, Albers et al. report that 50:50 ratio of 9- and 10-CLA glycerides beneficially enhances the protective antibody levels to hepatitis B. Interestingly, supplementation with CLA (50:50 ratio of 9- and 10-CLA) causes an increase in plasma IgA and IgM but a decrease in plasma IgE in human. Moreover, CLA supplementation also reduces the pro-inflammatory cytokines, such as TNF-α and IL-1β, while it increases the anti-inflammatory cytokines, such as IL-10 (Song et al. 2005).

4.4 Bacteriocins and Bacteriocin-Like Peptides and Proteins

Bacteriocins are general terms of proteins or polypeptides exhibiting antimicrobial activities, which are produced by a large amount of Gram-positive (+) and Gram-negative (−) bacteria. Bacteriocins are different from antibiotics. The major difference is that bacteriocins possess antimicrobial activities toward closely related strains; however, antibiotics have a wider antimicrobial activity spectrum. Recently, the production of bacteriocins from lactic acid bacteria (LAB) has gained great attention due to most LAB possessing the generally regarded as safe (GRAS) and qualified presumption of safety (QPS) status. Bacteriocins produced from LAB can be used as a food preservative agent in food industry and as a therapeutic agent in medicine (Ahmad et al. 2017).

4.4.1 Classification of Bacteriocins

The number of bacteriocins from LAB continues to increase. Bacteriocins from LAB are usually can be classified into four classes. At the beginning, Klaenhammer proposes to divide bacteriocins from LAB into four categories based on the diversity of their structure and physicochemical property, which includes Class I

(lantibiotics), Class II (non-lantibiotics), Class III (large heat-labile murein hydrolases), and Class IV (the lipid- or carbohydrate-containing bacteriocins) (Klaenhammer 1993). Later, with the most recent developments of bacteriocins, Heng et al. (2007) renew this scheme by reclassifying the Class III bacteriocins as bacteriolysins and the Class IV bacteriocins as circular bacteriocins (Table 4.2).

4.4.1.1 Class I Bacteriocins

Class I bacteriocins, also named as lantibiotics, are small peptides (<5 kDa) with the lanthionine and methyl-lanthionine in their primary structure. Recently, about 60 lantibiotics are isolated, and most of them from LAB. These bacteriocins are usually posttranslationally modified and stable to heat. They exert antimicrobial effects generally by targeting the cell wall of pathogens, especially Gram-positive bacteria (Ahmad et al. 2017).

Among them, nisin, a Class I lantibiotic from *Lactococcus lactis*, has been widely investigated. Until now, nisin is the only commercialized Class I bacteriocin that has been worldwide utilized in the food industry. Nisin is active against highly pathogenic and food spoilage microorganisms, such as *S. aureus* and *L. monocytogenes*. Nisin is extremely potent against its target bacterial strains even at a nanomolar level. Nisin has two known killing mechanisms. On the one hand, even though at lower concentrations, nisin could kill target bacteria through inhibiting enzyme activity. The specific mechanism underlying its action is mainly because nisin binding to lipid II prevents lipid II transporting peptidoglycan subunits from the cyto-

Table 4.2 Classification of bacteriocins

Class	Features
I	Lantibiotics, small (<5 kDa) peptides containing lanthionine and 3-methyl-lanthionine
Type A	Elongated amphipathic structures
Type B	Globular and compact structures
Type C	Multicomponent
II	Small (<10 kDa), nonmodified peptides (non-lantibiotic and noncyclic)
Type IIa	Pediocin-like peptides, possessing antilisterial activity
Type IIb	Two-peptide bacteriocins
Type II	All single-peptide nonmodified bacteriocins that do not fulfill the criteria of type IIa or type IIb
III	Large (>10 kDa) bacteriocins
Type IIIa	Bacteriolysins (bacteriolytic enzymes)
Type IIIb	Non-lytic bacteriocins
IV	Cyclic bacteriocins

Tsvetanka et al. (2018)

plasm to the cell wall, which leads to block the biosynthesis of cell wall, eventually causing cell death. On the other hand, at higher concentrations, the nisin-lipid II molecule complex could induce to form pores in the cell membrane of target bacteria, which damages to the cell physiological activities and function, eventually leading to cell death. In conclusion, the binding of nisin with lipid II is the critical molecular mechanism to induce the target bacterial death, involving the blockage of cell wall synthesis and the disruption of the cell membrane (Breukink et al. 1999; Wiedemann et al. 2001; Hsu et al. 2004).

4.4.1.2 Class II Bacteriocins

Class II bacteriocins are heat-stable, small (<10 kDa), non-lantibiotics, or nonmodified or pediocin-like antibiotics, transported by ATP-binding cassette transport systems. Among them, pediocin PA-1/AcH is the only commercialized Class II bacteriocin that has been worldwide utilized in the food industry. Generally, Class II bacteriocins perform their antimicrobial effects by increasing the membrane permeability of the target bacteria, which subsequently leads to the leakage of cytoplasmic substances and eventually cause cell death (Ahmad et al. 2017).

4.4.1.3 Class III Bacteriocins

Class III bacteriocins are large (>10 kDa) peptides, heat-labile lytic bacteriocins, and non-lytic bacteriocins, such as zoocin A, lysostaphin, helveticin J, and helveticin V (Joerger and Klaenhammer 1986; Vaughan et al. 1992). Generally, the lytic bacteriocins perform their antimicrobial effects by lysing the cell wall of bacteria.

4.4.1.4 Class IV Bacteriocins or Cyclic Bacteriocins

These bacteriocins, such as plantaricin S, leuconocin S, and uberolysin, contain lipid or carbohydrate moieties; therefore, they are sensitive to glycolytic or lipolytic enzymes (Wirawan et al. 2007).

4.4.2 Applications of LAB Bacteriocins

LAB have been extensively used in fermented foods due to their beneficial effects on promoting the nutrition, organoleptic qualities, and shelf life of foods. Recently, bacteriocins produced by LAB have attracted a lot of interest in both food and pharmaceutical industries (Ahmad et al. 2017).

4.4.2.1 In the Food Industry

In the food industry, bacteriocins exert huge potential in the food preservation. They can work efficiently either alone or in combination with other preservative methods, which is known as hurdle technology. Until now, only two bacteriocins, nisin and pediocin PA-1, have been commercialized for application in food industry. However, a large amount of research about LAB bacteriocins also provide promising perspectives for their usage as food biopreservatives (Cleveland et al. 2001).

There are several utilization ways where bacteriocins can be applied in food systems: direct inoculation of bacteriocin-producing LAB, addition of the bacteriocin, and utilization of bacteriocin-producing LAB-fermented product (Schillinger et al. 1996).

The effects of bacteriocins in food preservation have been widely studied, including the biopreservation in dairy, egg, vegetable, and meat products. Among them, nisin A and nisin Z have been proven to be highly effective against microbial contamination in food. It has been reported that the Class IIa bacteriocins are potential against the highly pathogenic and foodborne *L. monocytogenes* in ready-to-eat refrigerated food products (Calo-Mata et al. 2008). When using the pediocin PA-1/AcH producing LAB as starter culture for producing the fermented pork sausage, it also can effectively prevent *L. monocytogenes* contamination (Kingcha et al. 2012).

In addition, incorporating bacteriocins into the food packaging film or surfaces has also been proved efficient on food preservation (Galvez et al. 2007). For example, incorporation of pediocin PA-1/AcH into packaging film could significantly reduce *L. monocytogenes* on the meat surface (Woraprayote et al. 2013).

However, there are still some serious problems limiting the large-scale application of LAB bacteriocins, such as high producing cost and low efficiency against Gram-negative foodborne pathogens. In order to solve these drawbacks, hurdle technology through combining bacteriocins with other preservative methods could be adopted.

4.4.2.2 In Pharmaceutical Industries

Recently, there are a large number of ongoing research about the clinical application of bacteriocins. It has been proved that some bacteriocins are potent against Gram-positive human and animal pathogens, including multidrug-resistant (MDR) pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) strain and vancomycin-resistant *Enterococcus faecalis* (VRE) strain (Galvin et al. 1999; Okuda et al. 2013). For example, it has been reported that lacticin 3147 is active against the MRSA and VRE strain (Galvin et al. 1999). Additionally, it has been explored that nisin could efficiently prevent bovine mastitis. Another advantage of using bacteriocins instead of conventional antibiotic for therapy is due to bacteriocins which are usually friendly to the natural gut microflora. Furthermore, in comparison with conventional antibiotics, bacteriocins are simply biosynthesized through bioengineering to either increase their activity or specify target

microorganism. All these advantages of bacteriocins make it possess potential application prospect in therapeutic and pharmaceutical industries.

4.5 Other Metabolites

4.5.1 Vitamins

Importance of vitamins to our health has been well-documented, and thus extensive efforts have been paid on various methods to produce them effectively *in vivo* or *in vitro*. According to different solubilities in water, vitamins can be simply classified as soluble (V_B) and insoluble (V_K and V_E). Previously, milk mixed with LAB has been reported to show unique nutrition in vitamins, heating up the area of manipulating benign bacteria to yield vitamins. Among vitamin group, what LAB is able to secrete mainly includes folic acid (B_9), riboflavin (B_2), cobalamin (B_{12}), and V_K, with which, thereby, this review mostly involve. The main point to this part lies on the synthesis of vitamin and its association with disease.

4.5.1.1 V_{B2}

Riboflavin, prerequisite for two electron carriers (flavin mononuleotide, FMN, and flavin adenine dinucleotide, FAD) functioning in oxidation reactions, is contained ubiquitously in many kinds of foods, like eggs, meat, cereals, and some vegetables and is unable to synthesize by human (Bacher et al. 2000; Massey 2000; Burgess et al. 2004). Although food resource is rich enough, deficiency of riboflavin still occurs and leads to a series of inflammation in the mouth, lips, skin, and mucosa, which also promotes studies on strategy in producing riboflavin by bacteria, especially LAB (Mack et al. 1998). Given that the specific LAB species or culture conditions were usually required for yielding large amounts of vitamin, gene operators and enzymes involved in the synthesis of riboflavin in LAB have aroused more and more attention among researchers. Substantial riboflavin overproduction was described when all four biosynthetic genes (*ribG*, *ribH*, *ribB* and *ribA*) were overexpressed simultaneously in *Lactococcus lactis* subsp. *cremoris* strain NZ9000 containing pNZGBAH (Burgess et al. 2004). More interestingly, nisin also played a motivator for expressing riboflavin (Burgess et al. 2004). Generally speaking, in terms of biosynthesis of riboflavin, a complete version starts from its precursors, GTP and ribulose-5′-phosphate, then follows with seven enzymatic steps (though a little different in bacteria and fungi), and finally ends with bioactivation aided by flavokinase/FAD synthetase (Stahmann et al. 2000). Further researches into mechanism or signal of producing riboflavin would enable us to see a clear picture of nutrition interaction of LAB and host.

4.5.1.2 V_{B9} (Folic Acid)

Folic acid takes active part in a series of metabolic processes, including biosynthesis of amino acids, some vitamins, and methyl group, and activities of DNA in the development of growth, thereby exerting huge effect on human body (Penailillo et al. 2015). Unfortunately, humans are devoid of generating folic acid in vivo, which leads to a demand for dietary intake. Previously, considering the economy and convenience, engineered bacteria, especially LAB, were reported to function as the tool to produce folic acid in fermented foods, which provided a novel solution to the deficiency of folic acid in some foods (Wegkamp et al. 2007). On the other hand, increased need for folate production has been met by impressive researches on the mechanism behind folate synthesis and transportation. In terms of transportation, it is important to know receptor uptake. Generally speaking, folate was modified by intestinal glutamyl carboxy conjugase from polyglutamate to monoglutamate and absorbed with the aid of megalin, reduced folate carrier, and other folate-binding proteins in different parts of organisms (Holm et al. 1980). In particular, affinity and efficiency of carrier for folate are complicated, associated with species and amount, as well as the phenotype of folate (5-methyl-tetrahydro folate in dominance). For generation of folic acid, producing folate de novo requires involvement of 6-hydroxymethyl-7, 8-dihydropterin pyrophosphate (DHPPP), para-aminobenzoic acid (PABA), dihydrofolate (DHF), tetrahydrofolate (THF), 7,8-dihydropteroate (DHP), and guanosine triphosphate (GTP), among which the first two act as precursor and the last provides necessary energy (Rossi et al. 2011). However, it is pitiful that most strains of LAB are defective in some key enzymes to produce important materials, such as PABA. Moreover, other studies on function of folate reveal that germ cells need folate to proliferate and MR1 enables MAIT cells to receive microbial VB metabolites like folate, sparking further interest in exploitation of folate (Kjer-Nielsen et al. 2012; Walker 2016). In short, strategy of synthesis folic acid via LAB is fruitful, but how to refine condition and amount of production, how to extend strains of LAB with ability to generate folate, and finally how to accelerate absorption and promote interaction of folate produced by LAB with host still remain to be answered. Continued and assiduous efforts to decipher specific pathway of digestion for folate and their role in host metabolism could deepen our understanding of probiotic character of LAB.

4.5.1.3 V_{B12}

Vitamin B12 (cobalamin) was made up of four pyrrole rings and a cobalt atom, which also outstands itself for being the only vitamin containing metal atom (Birn 2006). The involvement of V_{B12} in two metabolic processes in vivo (one is production of methionine with homocysteine assisted by 5-methyltetrahydrofolate (5-MTHF) and another is formation of succinyl-CoA converted from methylmalonyl-CoA with the help of methylmalonyl-CoA mutase) established its fundamental role in human health, especially for severe anemia disease treatment (Gille and Schmid

2015). However, mammals, humans included as well, although possess ability to activate different types of V_{B12}, are incapable to synthesize V_{B12}, leading to dependence on food intake. Apart from various food resources, such as meat, eggs, and fish, synthesis of V_{B12} mostly lies in microorganism, which as a catalyst fuels resurgence of applying probiotic bacteria, like LAB, to generate V_{B12} (Belaiche et al. 1987). Given that V_{B12} is precious and limited in the human body, absorption and metabolism of V_{B12} attract much attention of researchers. It is now known that human body contains two main receptors for V_{B12}, intrinsic factor-B12 (IF-B12) receptor and cubilin–amnionless (AMN) complex, but in serum, V_{B12} will be combined with transcobalamin (TC) and haptocorrin (HC), suggesting the transportation of V_{B12} (Birn 2006). But, when it comes to dysfunction of digestion, there are some debates. Cohen, H. claimed that, in contrast to common sense of atrophic gastritis and achlorhydria associated with a dysfunction in food V_{B12} absorption, this connection was not always true, and instead the malabsorption actually resulted from more than one gastric places, and one of them had nothing with gastric atrophy or achlorhydria by the experiment of 19 volunteers to examine gastric histology and function (Cohen et al. 2000). On the other hand, some researchers are concerned with the impact of V_{B12} on gut microbiome community and interaction of bacterial host since some intestinal bacteria indeed produce small amount of V_{B12} (Allen and Stabler 2008; Girard et al. 2009). Degnan et al. (2014a) found that most human gut microorganisms were very likely to compete with the host for V_{B12} and relevant cofactors (corrinoids) (Degnan et al. 2014b). More interestingly, synthesis of V_{B12} was reported to have possible implication with aging in mouse (Degnan et al. 2014b). In a word, metabolism and absorption of V_{B12} are recognized as significant in human health, and further researches into cross talk of intestinal bacteria with host on dietary V_{B12} could unravel the secret of malabsorption to V_{B12} and thus offer promising solution to relative disease with deficiency of V_{B12}.

4.5.1.4 VK

Vitamin K, comprising of two forms, that is, phylloquinone (vitamin K1) mostly from green vegetables and menaquinones (vitamin K2) largely obtained from animals and gut bacteria, is known to have close relationship with intracranial hemorrhagic disease in baby and osteoporotic fractures due to its role in carboxylating glutamic acid to γ-carboxyglutamic acid (Gla), which exerts their effects on blood coagulation and tissue calcification by combining with matrix Gla-protein (MGP) and osteocalcin (Shearer 1995; Feskanich et al. 1999; Parker et al. 2003). With respect to resource of VK2, LAB has been reported to produce certain amount of menaquinone and aroused interest of dairy industry, owing to increased nutrition in fermented products and hence profitable expectation (Morishita et al. 1999). Besides, not only LAB produce menaquinone; menaquinone also, in turn, has influence on respiration metabolism of LAB (Pedersen et al. 2012). Specifically, menaquinone as well as heme can act as stimulator of respiration, by means of releasing electrons, for some species of LAB, thereby promoting growth and yield (Pedersen

et al. 2012). However, given the population of study in gut bacteria, recently there is a trend to take menaquinone produced by intestinal bacteria as a maker to represent health status of human (Karl et al. 2015). Karl J.P studied fecal menaquinone material of fat adults to figure out possible relationship of menaquinone material from feces, community composition in gut bacteria, and signals of cardiometabolic disease, and in the end, the result showed that concentration of menaquinone in feces was overwhelmingly decided by a few kinds of gut bacteria (Karl et al. 2015). In agreement with this view, Fukumoto et al. showed that a kind of precursor of VK2, namely, 1,4-dihydroxy-2-naphthoic acid (DHNA), had the potential to activate the aryl hydrocarbon receptor (AhR) characterized by suppressor of inflammatory bowel diseases (IBDs), thus suggesting important link between VK2 and colitis (Fukumoto et al. 2014). Notably, since VK2 also participates in formation of microorganism membrane, now some researchers tried to find new antibiotic targeting at destroying bacterial membranes, for example, the discovery of lysocin E, claimed to be an effective killer for bacteria owing to its capacity to influence VK2 in membrane (Hamamoto et al. 2015). In a word, further study in distribution of dietary VK2 between host and gut flora and possible application of VK2 to biomarkers indicating status of human body will renew our understanding of nutrition absorption in gut.

4.5.2 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a by-product of O_2-dependent metabolic pathways of many LAB. There are significant differences on the amounts of H_2O_2 production of various LAB, which is in a range of 1.01–15.50 μg/mL H_2O_2 (Aslim and Kilic 2006).

H_2O_2 is an oxidizing agent, which is toxic to catalase-negative bacteria such as most anaerobic microorganisms. It is well-known that the H_2O_2 formed by LAB plays bacteriostatic effect on Gram-positive bacteria and bactericidal effect on Gram-negatives. This trait may be desired or undesired depending on the environment. It has been reported that H_2O_2 formed by LAB exerts antibacterial role in foods and feed (Krockel 2011).

In addition, H_2O_2 formed by LAB plays an important effect on controlling the vaginal health. It has been reported that approximately 80% of the strains of vaginal origin produce H_2O_2, mainly including *L. crispatus* and *L. jensenii* vaginal isolates (Antonio et al. 1999; Kovachev 2018).

Many studies have indicated that the presence of H_2O_2-producing vaginal *lactobacilli* may prevent infection with HIV-1, herpes simplex virus type 2, and pathogens associated with bacterial vaginosis (BV) (Conti et al. 2009; Matu et al. 2010). Moreover, H_2O_2-producing *lactobacilli* also might exert anti-vaginal cancer effects by interacting with superoxide anion, hydroxyl radicals, and hypochlorous acid.

4.6 Conclusions

It is currently getting evident that LAB belongs to probiotics and is applied to a wide range of industries owing to its safe and economical qualities. Notably, recent researches suggest that it is various secreted metabolites (such as short fatty acids, vitamins, and γ-amino butyric acid) that enable LAB to exert important functions and involve in host dynamic physiological activities and hence profoundly influence host health through either directly metabolites or indirectly activation of metabolic signals. These findings will expand our knowledge of beneficial effects of fermented foods and at the same time how our diet will balance our outer and inner physiology. For example, the widespread application of fermented foods containing LAB and other probiotics already revolutionizes our dairy industry. Further studies into details of metabolites secretion by LAB and also following transportation and distribution in the body will, undoubtedly, unravel the confusion of cross talk between host and symbiosis, which in the end allow us to think creatively about bacteria and some related disease, like obesity, diabetes, and colitis.

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Chapter 5
Environmental Stress Responses of Lactic Acid Bacteria

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Contents

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W. Chen, A. Narbad, *Lactic Acid Bacteria in Foodborne Hazards Reduction*,
https://doi.org/10.1007/978-981-13-1559-6_5

Abstract Lactic acid bacteria (LAB) are often used to produce fermented foods. LAB usually grow in "moderate" environmental conditions and commonly encounter stress conditions like changes in pH, temperature, production, storage, and others (Kim NR, Jeong DW, Ko DS, Shim JH, Intl J Biol Macromol 99:594–599. https://doi.org/10.1016/j.ijbiomac.2017.03.009, 1997). These condition changes may lead to poor growth rate or even survival of the bacteria. Stress responses were of great importance for microorganism; they always continually change with temperature and osmotic pressure in the environments (Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL, J Clin Microbiol 40(3):1001–1009. https://doi.org/10.1128/jcm.40.3.1001-1009.2002, 2002). There are various kinds of stress factors, which include physical, chemical, or biological and others. LABs are exposed to these stresses during fermentation, for example, low temperature, high H_2O_2, and low pH (Kurz M, Saline Syst 4:6. https://doi.org/10.1186/1746-1448-4-6, 2008; Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, Stanton C, Dinan TG, Cryan JF, Biol Psychiat 82:472. https://doi.org/10.1016/j.biopsych.2016.12.031, 2017; Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, Griffen AL, J Clin Microbiol 40(3):1001–1009. https://doi.org/10.1128/jcm.40.3.1001-1009.2002, 2002). LABs have the stress-sensing systems to activate defenses, permitting the bacteria to acclimatize the harsh conditions or environmental changes. In LAB, DNA-repairing mechanisms can be also characterized as responding to oxidative stress and acid stress.

Keywords Acid Stress · Bile Stress · Osmotic Stress · Oxidative Stress · Cold Stress

5.1 Response of Lactic Acid Bacteria to Acid Stress

5.1.1 Introduction

Lactic acid bacteria (LAB) are often used to produce fermented foods. LAB usually grow in "moderate" environmental conditions and commonly encounter stress conditions like changes in pH, temperature, production, storage, and others (Kim et al. 2017). These condition changes may lead to poor growth rate or even survival of the bacteria. Stress responses were of great importance for microorganism; they always continually change with temperature and osmotic pressure in the environments (Becker et al. 2002). There are various kinds of stress factors, which include physical, chemical, or biological and others. LABs are exposed to these stresses during fermentation, for example, low temperature, high H_2O_2, and low pH (Kurz 2008; Burokas et al. 2017; Becker et al. 2002). LABs have the stress-sensing systems to activate defenses, permitting the bacteria to acclimatize the harsh conditions or environmental changes. In LAB, DNA-repairing mechanisms can be also characterized as responding to oxidative stress and acid stress.

The stress-resistance systems of LAB were sorted into three classes: (1) specifically induced by a sublethal dose of the stress, (2) adapting to one stress condition

that allows the cell to resist others in general systems, and (3) stationary-phase-associated stress response, which can induce plenty of regulons that are designed to conquer some stress conditions (Erny et al. 2015; Tsou et al. 2015; Beales 2004; Keijser et al. 2008; Becker et al. 2002).

5.1.2 The Metabolic Responses to Acid Stress by LAB

More studies described the mechanisms underlying the capacity of LAB with acidification. Many references concentrated the resistance of LAB to physical damages with acidic condition. For instance, a few of oral bacteria exhibits strikingly differences in the ability to acidification (Ayres 2016; Beales 2004). In these experiments, all streptococcal species found similar release of magnesium at pH 4.0 conditions. However, *Lactobacillus casei* illustrated damage below of pH 3.0, which had more resistance. Experiments assessed the proton movement on the membrane of *L. casei*. At the same time, the addition of ATPase inhibitor can cause increase of the proton permeability of *L. casei*, indicating that the proton entered the cell membrane, while the outflow process involved the proton-translocating ATPase (F_1F_0-ATPase). F_1F_0-ATPase can utilize ATP to pump several protons from the cytoplasm in the process of glycolysis. Many studies have shown that some F-ATPase activity in microorganism have their own ability to transfer protons from the cytoplasm to maintain the homeostasis of pH. LABs have the F-ATPase that may have more acid resistance during low pH conditions (Uchihashi et al. 2011). The F-ATPase of LAB is significant because of its good competitiveness relative to other organisms and basic level of inherent acid resistance provided by the enzyme.

LAB could also produce cyclopropane-containing fatty acids (CFAs) in variable conditions, for example, dehydrosterculic acid (C19cyc9) and lactobacilli acid (C19cyc11). In the case of CFA synthase, methyl group can be transformed from S-adenosyl methionine to fatty acids, which can produce CFAs. Bender et al. found that the acid-adapted cells of *L. casei* ATCC334 are much more than those of control cell, which may increase the levels of CFAs and saturated fatty acids at the cost of UFAs. These results showed that CFA production, occurring in *L. helveticus* and *L. sanfranciscensis*, may be part of the rigid response in *L. casei*. CFAs occur in *L. helveticus* and *L. sanfranciscensis*. Meanwhile, a *rel*A gene involves the starvation response of *Streptococcus mutans* and the production of CFAs under culture condition. In this condition, *rel*A mutant strain showed the larger acid tolerance compared with the parent strain, which was the major effects of the mutation, following growth in biofilms (Bender et al. 1986).

In addition to abovementioned acid tolerance mechanisms of LAB, some other mechanisms of LAB cope with the influence on the damage mediated by acid and improve degressive internal pH values. For example, releasing the ammonia to increase pH value is thought to be a mechanism of acid resistance that distributed evenly (Budin-Verneuil et al. 2006). In common conditions, arginine and amino acid agmatine as parts of sources of ammonia. The release mechanism of ammonia contained the system of arginine deiminase, and it needs to depend on the enzymatic activity: ornithine carbamoyltransferase, arginine deiminase, and carbamate kinase

(Champomier Vergès et al. 1999). Arginine is across cells membrane assistances an arginine-specific porter, as part of an arginine-ornithine antiporter system. Arginine can be converted into ammonia and citrulline when it was cleaved by arginine deiminase. Meanwhile, citrulline was delaminated and splitted by ornithine transcarbamylase to ornithine and carbamoyl phosphate. Carbamate kinase can cut carbamoyl phosphate that would become carbon dioxide and ammonia in the substrate-level phosphorylation (Davis et al. 1986; Champomier Vergès et al. 1999). Finally, these involved systems convey the moles of ammonia into cells and an ATP through a series of energy formation reactions.

LAB exploits L-malate, because it can be converted into carbon dioxide by malolactic fermentation pathway (MLF). Within this pathway, L-malic acid will release carbon dioxide by malolactic enzyme and then form one molecule of lactic acid and carbon dioxide, which could be transformed into bicarbonate by carbonic anhydrase (Konings et al. 1997). It is an antiporter system that lactic acid was produced and a molecule of L-malate was used. Since MLF pathway can be found in many LAB, for example, it was used in some wine industry and so on. It is apparent that MLF pathway is associated with protective buffering for the host bacteria. (Konings et al. 1997). MLF is widely distributed among LAB and distinctly within its strain levels. During the fermentation of sugar, LAB could be respect to the ability to grow with alcohol and at low pH (Vrancken et al. 2009).

Numerous studies concerning with LAB have described main growing period of stress response to acid. No well-defined about lactic acid bacteria deploy their acid tolerance, because of significant difference of its species and strain at the same media composition and culture conditions (Beales 2004; Konings et al. 1997). Hence, the transcriptional profiling and proteomics were used in combination to illustrate the changes of LAB under in the acid stress responsive genes. Importantly, the guanidine nucleotide pools of LAB especially affected by starvation, and carbon flow was disrupted by Embden–Meyerhof–Parnas (EMP) system.

5.1.3 Signal Transduction

(p)ppGpp is a perfect position that can influence the variation of cells and the port of conversion, carbon flow, and phosphate pools. Obviously, in different species and strains, protein production is influenced by RelA or its equivalent loss (Magnusson et al. 2005). A fewer reported in the literature about above theory of LAB. For example, Rallu et al. once reported *Lactococcus lactis* responds rapidly to acidification involving pppGpp (Rallu et al. 1996, 2000). Budin verneuil et al. suggested that there is a deficiency in the protein in relA, guaA(GMP synthase), and pstS(phosphate transporter) with more *L. lactis* mutant strains compared with the parent strain (MG163) (Budin-Verneuil et al. 2007). For instance, RecA (DNA-repair-recombination protein), pyruvate carboxylase, CTP synthase, glutamyl–tRNA synthetase, R30S ribosomal protein S1, and the subunit of DNA polymerase could overlap the mutant strains, playing important roles at the acid conditions. dnaN, similarly, a whole transcriptional studies about the relA mutant strain of *S.mutans* refer to 50 transcripts, involving the strict response to RelP- and RelQ-mediated

RelA-independent stress response (Nascimento et al. 2009). Certainly, RelA also impact acid tolerance of *S.mutans*, when forming biofilms and participating in the quorum sensing mediated by AI-2(Lemos et al. 2004).

More literatures show that LAB in an intense area, which could explore two-component signal sensing and response circuits. The general concept about two-component regulatory systems (TCSs) is that it can regulate extensive bacterial reaction under the external stimulus (Gao and Stock 2009; Krell et al. 2010).

More literature indicates that TCSs were related to the control of acid stress response in laboratory (Cui et al. 2012). For example, in the case of *S. mutans*, multiple TCSs affect the sensitivity to acidification (Li et al. 2002). It has been reported that HK03 and RR03 protein sequences are the basis for BLAST search of *S. mutans* UA159 genome sequence (Li et al. 2002). Levesque et al. (2007) reported an exhaustive study about 14 recognizable TCS pairs of *S. mutans* (Levesque et al. 2007). The results illustrated that tcs-2, the homologue of CiaRH system, tcs-3, same as the scnrk-like system, tcs-9, were all involved in a certain degree of acid resistance. The new studies by scholars found that the VicRK TCS of *S. mutans* was the acid resistance of *S. mutans*, while the loss of vicK gene affected 89 transcripts in the microarray analysis of the vicK mutant strain (Senadheera et al. 2009). Other studies involved all 14 transducers/kinase pairs of mutations in the stress reaction (Biswas et al. 2008; Kawadamatsuo et al. 2009). These studies also suggest that the expression of the TCS must be carefully explained, because at least some of them do overlap (Chong et al. 2008). With the continuous development of information, TCSs responsiveness - adjustment partner's DNA binds to theme (Senadheera et al. 2005). There is no significant distinguish about TCSs, which regulate the acid conditions.

Meanwhile, a study with Group B *Streptococcus* (GBS), strain V/R 2603, has been published, and the global transcriptional analysis of biological growth with a pH of 7.0 and 5.5 is reported (Santi et al. 2009). Similar studies on gene transcription found that about 300 genes were upregulated in pH 5.5 and contrasted with pH 7.0, and 61 genes were downregulated at pH 5.5. Genes expressed in acid growth involve all major metabolic and stress response pathways. In their study, the genes that focused on the pH were controlled by the CsrRS TCS, and about 90% of the downregulated genes and nearly 60% of the upregulated genes were related to the CsrRS. GBS will be from the mother's vagina (acid) transferred to the baby's lungs, which may show toxic factor upregulation of invasive phenotype and GBS, including surface protein BibA, this is a kind of GBS vaccine candidates with ph response ability (Santi et al. 2009).

5.2 Responses of Lactic Acid Bacteria to Bile Stress

5.2.1 Introduction

LAB being the most representative probiotics is used for the production of fermented dairy, vegetable-based food, and wine. Bile tolerance, the most crucial property, commonly conformed to the capacity of the bacteria to grow which functions

as probiotics (Macpherson et al. 2016; Marchesi et al. 2016; Fetissov 2017; Hamon et al. 2011).

Normally, bile contains cholesterol, bile acids, phospholipids, water, and pigment biliverdin (Patel et al. 2010). It always produces a yellow or green solution in pericentral hepatocytes from the liver of mammals. Bile in the liver could eliminate some biological substances synthesized from cholesterol. The process included the generation of bile flow, and its physiological function is to facilitate the absorption of lipophilic compounds from food (Hu et al. 2015). Bile also has a vital role in the establishment of the intestinal microbiota in humans. Generally, bile could disturb the function of cell membrane in LAB and bifidobacteria (Xiong et al. 2017). Studies on the bile stress response in LAB and bifidobacteria have shown that bile resistance can lead to an integration of multilateral responses, protecting cell membrane resist from bile acids. This process involved in the restoration or degradation of proteins, elimination of the oxidative stress, facilitation of DNA repairing, and enhancement of energy generation by upregulating sugar metabolism (Jie et al. 2016; Patel et al. 2010).

5.2.2 The Mechanism Responses to Bile Stress in Lactic Acid Bacteria

LAB can affect host beneficially by enhancing its intestinal microbial balance. Therefore, LABs have the ability to be resistant to the enzymes and the digestion process. When bacteria enter to the intestinal tract, bile will reach the duodenal section of the small intestine to reduce the bacteria survival. Bile acid is one factor that reduces strain survival in culture conditions. Bile acids can be flip-flopped by lipid bilayer, which increases the tensile strength of the membrane. Hence, plenty of bile acids will bring to bear on cells if it is too much (Bandyopadhyay and Moulik 1988). When the concentration of bile acid becomes higher, the apparent proton conductance and membrane osmosis ability will be disturbed (Martoni et al. 2008).

The ability to survive in bile exposure can be considered as a pivotal factor to select probiotic strains. Membrane characteristics and cells of bacteria will be influenced by bile, which contain dissolution, DNA lesion, acid, oxidative, and osmotic stresses. Therefore, these factors, such as bile, oxidative, acid, detergent, and salt stresses should be cosidered in the studies of bile tolerance. The survival mechanisms of bile tolerance are still indistinct, but the several genes and molecules refer to bile have been identified in lactobacillus (Ai et al. 2008).

Bile salt serves as biological surfactant, which damages the cell membrane of bacteria, leading to cell leakage and cell apoptosis. Hu et al. reported that *L. plantarum* was resistant to 0.3% bile content and put the bile tolerance into future functional assessment (Hu et al. 2015). More studies suggested that the intact membrane

of cell plays an important role in defensing bile (Begley et al. 2005). Lipids play a vital role for keeping the structure of cell membrane, such as fatty acid compound of LAB, which is crucial in the bile tolerance (Küllenberg et al. 2012; Hu et al. 2015).

The hydrolysis of bile salts and the resistance of bile are separated in bile, and bile acids often have potential toxicity and cholesterol metabolites. The use of wild type and *bsh* gene mutation combination in the bile brine solution and bile tolerance of probiotics can effectively reduce the damage of bile salts. The BSH activity was reduced in *L. amylovorus*, as well as the the growth rate of bile salts in existence. However, cells are easily affected by bile because of the mutational *bsh* in *Listeria monocytogenes* and *L. plantarum* (Jones et al. 2008).

Bile acids could be conjugated with amino acids to produce the conjugated bile salts (CBA) and then emulsification and solubilization of lipids. CBAs demonstrate antimicrobial activity by interfering with the cell membrane and homeostasis (Begley et al. 2005). However, LABs have in particular defense mechanisms, which resist these harmful behaviors. The hypothesis is that bile salt hydrolase can conjugate bile salts and may improve bile tolerance and bacteria survival in the gut (Lee et al. 2008). Jarocki et al. suggested that bile acid can also be released by bile salt hydrolase reaction, forming micellars on the membrane of *Bifidobacterium* under bile pressure (Jarocki et al. 2014).

5.2.3 Bile Salt Hydrolases of LAB

Bile salt hydrolase (BSH) is so crucial in the cholesterol-removing effect of LAB. It can hydrolyze conjugated bile salts to amino acid and connect bile salts. *L. casei* has been reported that lacking of *bsh* gene may highly sensitive to bile salt stress (Wu et al. 2012). Much more literatures suggested that probiotics have evolved BSH to deal with bile salt stress (Wang et al. 2011; Allain et al. 2017). BSH is a vital enzyme for eliminating cholesterol and catalyzing the conjugated or deconjugated bile salts to free amino acids. Moser and Savage reported that *L. buchneri* JCM1069 has hydrolase activity metabolizing taurodeoxycholic acid rather than taurocholic acid (Moser and Savage 2001). These acids are usually made of taurine as their amino acid, but there are seven different positions in the steroid. Meanwhile, it's not relative between BHS activity and resistance to toxicity of conjugated bile salts in LAB (Moser and Savage 2001).

The mechanism of BSH is not well known. Studies have shown that bile salts can form protons, showing toxicity through the cells' interfaces, while BSH positive cells may protect themselves (Kurdi et al. 2006). The process can eliminate acidification by recycling and exporting the protons. BSH is so specific to certain types of bile that the duration of it can guarantee the survival of bacteria in a changing bile environment. For instance, *L. plantarum* WCFS1 and *L. acidophilus* NCFM have four and two *bsh* genes, respectively, which supported this theory (Patel et al. 2010).

5.2.4 Scope of Bile Salt Hydrolases of LAB

High cholesterol level is a main challenge for human health worldwide. However, probiotic-based oral therapy can efficiently reduce the cholesterol level of blood, and BSH activity has nothing to do with yield. *L. plantarum* CK102 isolated from human could reduce the levels of blood cholesterol, triglyceride, LDL-cholesterol, and free cholesterol in rats. The supernatant of *L. acidophilus* ATCC43121 has exhibited cholesterol-removing activity as well (Ahn et al. 2003).

The extract of bile salts produces amino acids, which are then used as carbon, nitrogen, and support sources. For instance, glycine could be hydrolyzed into ammonia and carbon dioxide. However, taurine is hydrolyzed into ammonia, carbon dioxide, and sulfate. Meanwhile, this process has been taken place by BSH-positive strains. The decompression of *L. Acidophilus* SNUL020 and SNUL01 was reported at a similar speed (Peschel 2002).

BSH cannot display deconjugated activity against the primary salts, when it was deconjugated to the secondary salts. Whether the bacteria express the resistance to bile via the accumulation of BSH remains unclear. However, it is assumed that the protonation of bile salts is toxic through the intracellular interface, while BSH positive cells may be protected by weaker nonconjugated cells (Taranto et al. 1997). This process can be explained that the acidification can be eliminated by restoring and exporting the protons. When the probiotics cells were microencapsulated to achieve the BSH decompression rate, *L. reuteri* microcapsules metabolize glycol and tauroconjugated bile salts at rates of 10.16 ± 0.46 and 1.85 ± 0.33 μmol/g microcapsule per hour, showing better acid tolerance (Tsou et al. 2015; Kim et al. 2017.

5.3 Responses of Lactic Acid Bacteria to Osmotic Stress

5.3.1 Introduction

LABs have often been exposed to adverse environmental conditions such as industrial processes, natural environment, and human infection. Osmotic stress is a prominent limitation, which can bring about a decrease in survival or growth and affect strains metabolic activities. Osmotic pressure is one of the main stresses encountered by LAB in an industrial environment such as cheese production, beer brewing, and yogurt-making process.

In the manufacture of some foods, LAB is used as a leavening agent. During the process of starter culture preparation, The LAB is exposed to adverse culture, affecting their viability and performance. LABs are confronted with extreme value of pH and osmotic stress conditions, which affect the survival of LAB negatively by dis-

turbing cellular viability. Probiotics such as *Lactobacillus* and *Bifidobacterium* are widely employed in yogurts, dietary adjuncts, and other health-related products. To survive and proliferate in gut, LAB may need to elevate the osmotic stress in the upper small intestine. Meanwhile, the osmotic pressure was significantly increased when human was infected with lactic acid bacteria. It is also caused by perspiration in skin infections.

In response to osmotic stress, the development of adaptive strategy is the key to the function of lactic acid bacteria in food fermentation. Therefore, the study of osmoregulation and adaptation of cells to changes in the external osmotic stress is so vital for us to understand its important industrial and medical aspects.

5.3.2 Fundamental Principles of Responses to Osmotic Stress in Lactic Acid Bacteria

In order to insure the direction of water flow enter the cell in the period of growth, the density of solute is much higher than the density demanded to metabolize for these cells. At the same time, all of these growing bacterial cells show high levels of outward expansion pressure, making the membrane close to the expanded polysaccharide wall. It is generally believed that maintaining invariable expansion is the driving power to expanding, growing, and dividing of cell. Changes of water activity in extracellular have a direct influence on the water activity in the cytoplasm, with the water flowing alongside the osmotic gradient. It is generally shown that bacterial cytomembrane shows high water permeability, but as for the majority of solutes, it is an effective barrier. Water could enter into and leave out the cell until the osmosis pressures on both sides of the semipermeable membrane reach equilibrium. The flow of water could cause swelling and bursting of the cell in the condition of hypotonic or dehydrating, shrinking, and plasmolysis in the condition of hypertonic. Water-selective channels, namely, aquaporins, embedded in the membrane, accelerating the transition of water. Such channels regulate the water flux in both directions in order to respond to a sudden penetration or drop. Aquaporins belong to the family of major intrinsic protein (MIP) of transporters ubiquitous. Glycerol facilitators and aquaglyceroporins are also included in the family, which also permits the transition of some small molecules, such as glycerol, other polyols, dihydroxyacetone, CO_2, urea, and ammonium.

To avoid undergoing detrimental conditions, microbiology has established an efficient and quick countermoves, in addition to a passive volume regulation. The swelling of bacteria is under control by regulating the osmotic activity of the pool of solutes in the cytoplasm, therefore enabling to adjust the water content via osmosis. This mechanism includes the recovery of swelling, which is one aspect of the most in-depth study of the reaction of LAB to osmotic pressure.

5.3.3 Regulatory Mechanisms in the Osmotic Responses of Lactic Acid Bacteria

The osmotic active compounds in the *Lactobacillus* family have dual functions in osmotic regulation cells. They not only play a role in keeping cell expansion but also protect biomolecules in vitro under pressure. Many bacteria can protect against osmotic stress through accumulation of glycine betaine, carnitine, and proline (Caldas et al. 1999). Research shows that the beneficial effects of glycine betaine of LAB, which discovered the *Rituxan* cloning and expression of betaine BetL intake system, significantly improved the *B. breve* UCC2003, the cloning and expression of liszt bacteria, and acid resistance and salt resistance (Sheehan et al. 2006, 2007). Another important function of compatible solutes is offset due to dry damaging effects of water loss. *L. plantarum* can be protected by glycine betaine during the drying processes (Kets et al. 1996). When *L. plantarum* ST-III was cultured in a chemically defined medium with 6% NaCl, glycine betaine significantly improved cell growth (Zhao et al. 2014). Transcriptomics data showed that under the existence of glycine betaine, the gene expression of carbohydrate transport and metabolism was significantly increased. This may be the resistance mechanism of *L. plantarum* ST-III to salt stress.

The mainly protective agent of sugar is recognized in the preparation of the dried LAB starters (Santivarangkna et al. 2008). It affects the vitality of the starter from the beginning to the end of the process. As mentioned above, some LAB responses to sugar and permeability are affected by the balance of intracellular and extracellular glucose concentrations. As a result, these compounds are present on both sides of the cell membrane and are in contact with the cytoplasmic protein inside. The accumulation of trehalose or the oligosaccharides of *Lactobacillus* was observed (Kets and Bont 1995; Prasad et al. 2003).

Excepting the regulation of intracellular solutes in hypertonic conditions, LAB rapidly changes the expression of some genes directly refer to the uptake of osmotic agents. Under the high osmotic stress, the stress response protein inhibited the molecular adjoint protein and protease to a certain extent, which was the main stress response system of the protein quality control of LAB. Transcriptional and proteomic studies showed that DnaK, GroEL, and GroES were involved in the reaction of lactate to salt stress (Kilstrup et al. 1997; Xie et al. 2004). The transcription of dnaK gene in *Enterococcus faecalis* was induced by hyperosmotic conditions too (Flahaut et al. 1996). Analogical observations were found in acidophilic siphonococcus and a variety of *Lactobacillus* under high salt conditions. (Fukuda et al. 2002; Prasad et al. 2003; De Angelis and Gobbetti 2004).

The variation in bacterial cell wall compositions with external osmolality is another important aspect of the osmoadaptation. High osmotic pressure affects lipid composition of bacterial membranes. These changes affect the osmotic activation curves of osmotic protective agent transporters operated in *L. lactis* by lipid-protein interactions. Modifications may also affect the permeability of the membrane. The

permeability reaction is in connection with the physical properties of the membrane. Compared with the growth of the standard MRS, the cells with the lower growth of polyethylene glycol have a more rigid structure, which refer to the increase of saturated/unsaturated ratio in the total pool of bacterial lipid (Tymczyszyn 2005). The increase of cyclopropane is mainly due to the modification of high-permeable *Streptococcu*s in the composition of membrane fatty acids (Guillot et al. 2000). In *L. bulgaricus*, the change of membrane performance was also related to the increase of sugar content in the whole lipid pool. In *Lactobacillus bulgarian*, the changes of membrane properties were also related to the increase of total lipid sugar content (Tymczyszyn 2005). The reaction of *Lactobacillus casei* to hyperosmotic conditions did not lead to prominent differences in the proportion of glycolipids/phospholipids (Machado et al. 2004). Nevertheless, individual glycolipids and phospholipids revealed several important changes. The small increase in the amount of glycolipids involved in the formation of liposomes may be especially related to the hydrophobicity of the cells shown in *Lactobacillus*. Some phospholipids were observed to increase significantly. The different content of cardiac phospholipids is considered as a key factor for bacterial infiltration (Romantsov et al. 2009).

Moreover, LAB can react to osmotic pressure by changing the properties of cell walls. The modification plays a major role in the industrial application of LAB under high permeability, because they can modify the sensitivity to cracking (Piuri et al. 2005; Koch et al. 2007). The retardation of *Lactobacillus casei* in high salinity was related to cell size observed by transmission electron microscopy and modification of cell membrane (Piuri et al. 2005). In addition, in *Lactobacillus*, short osmotic stress (with 4% NaCl 30 min) caused the induction of *murF* and *murG* genes (Xie et al. 2004) involving polysaccharide peptide biosynthesis.

5.4 Responses of Lactic Acid Bacteria to Oxidative Stress

5.4.1 Introduction

The imbalance between production of reactive oxygen species (ROS) and antioxidative mechanisms is oxidative. ROS and oxygen do harm to the biomolecules such as nucleic acid, protein, and lipids, damaging their biological functions. LABs are widely used as a starter culture in food fermentation, and it is vital to secure title of viable cells. During manufacturing process, LAB exposure to oxygen and reactive oxygen species contributes beneficial effects on human health, since it commonly functions as a probiotics. In the gastrointestinal tract, LABs encounter oxidative stress from oxygen gradients and the immune system, reducing viable cell counts (Bermúdezhumarán et al. 2008).

Gene expression, growing, and surviving of LAB were significantly affected by oxidative stresses, since the applications of LAB were restricted. The gene expression of LAB was reprogrammed by the stress; therefore, the physiology adapted to the new to survive.

5.4.2 Metabolic Responses to Oxidative Stress in Lactic Acid Bacteria

In *Lactococcus lactis*, the conversion of glyceraldehyde-3-phosphate is catalyzed to glycerate-1,3-biphosphate in glycolysis by glyceraldehyde-3-phosphate dehydrogenase. Among the most abundant proteins, the *gapB* gene is highly expressed by glycolytic pathway. The gapB gene has been probed in 2D gels, and when its cells are exposed to O_2, it presents as two spots with different isoelectric points but the same molecular weight (Melchiorsen et al. 2000). It was observed that the relative level of these two spots of *Lactococcus lactis* has changed in these circumstances: one is in a thioredoxin reductase mutant (trxB1) and another is in respiration metabolism. At the same time, both of these circumstances are related to oxidative stress (Vido et al. 2004, 2005). Thioredoxin–thioredoxin reductase system needs to remove reactive oxygen species (ROS), such as O_2, superoxide, H_2O_2, and hydroxyl radical, in order to avoid cysteine of the GapB protein oxidation before it is attacked. A mass of GapB of *Lactococcus lactis*, which can avoid glycolysis flux declining via the oxidation of GapB, still keep the whole activity of glycolysis (Boels et al. 2003). What's more, the growing of without texB1 slowed down under the condition of glutathione, cysteine, and pyruvate (Vido et al. 2005).

In anaerobiosis, pyruvate formate lyase (PFL) was catalyzed from pyruvate to acetyl-CoA. PFL is divided into two fragments in the shift from anaerobiosis to aerobiosis and then lead to its irreversible inactivation. Reducing the glycyl radical into glycine by PFL decativase could avoid cleavage (Melchiorsen et al. 2000). In addition to this, the alternative metabolic pathway induced to sustain acetyl-CoA productions. At the later stages, the gene that encoded pyruvate dehydrogenase compound (PdhABCD, PDHc) expressed (Jensen et al. 2001). The compound also acts as catalyst of the transformation of pyruvate to acetyl-CoA. In aeration conditions, flavoprotein has vital role in the growth of several bacteria. An NADH:H_2O of *Lactococcus lactis* that leads oxidase to overproducing generated a vast of acetate and acetoin, at the exchange of lactate, indicating that the enzyme can effectively promote the transformation of pyruvate to acetate (Hoefnagel et al. 2002).

Researches show that *Lactococcus lactis*, *E. faecalis*, and other LAB can activate cytochrome oxidase that is heme-dependent and establish a whole respiration chain (Winstedt et al. 2000; Duwat et al. 2001). The heme-dependent cytochrome oxidase CydAB is usually reported to have more affinity for O_2 than other cytochrome oxidases (Rezaïki et al. 2004). Meanwhile, the consumption of O_2 in the membrane decreases O_2 content in the cytoplasm and restricts the occurrence of ROS. Due to ROS reactivity, *Lactococcus lactis* and other breathing-permissive LAB demand regulate their heme library, yet permitting enough heme enables to activate the CydAB cytochrome oxidase (Rezaïki et al. 2004).

5.4.3 Regulatory Mechanisms in the Oxidative Stress of Lactic Acid Bacteria

LlrF (DNA-binding regulator) and LlkinF (sensor), involved in oxidative stress, were identified as two TCSs in *Lactococcus lactis* (O'Connell-Motherway et al. 2000). Inactivation of LlrF is more sensitive to peroxide, and only 9% of mutants survive when exposed to 4 Mm H_2O_2.It seems that a TCS called ScnRK in *Streptococcus mutans* could regulate some genes associated with stress, for example, TPX, which could encode mercaptan peroxidase (Chen et al. 2008).

In *Lactococcus lactis*, genome analysis showed the PerR gene and OhrR gene of *Bacillus subtilis*, related to peroxide stress response. In *Bacillus subtilis*, the PerR gene is associated with the iron-containing repressor family (Lee and Helmann 2006). Meanwhile, the PerR reacted with peroxide via iron through Fenton reaction, and what is more, $HO^{\cdot}$ oxidized histidine in location 37 or 91 in peptide sequence (Lee and Helmann 2006). Research showed a regulator similar with PerR in *E.faecalis* could cause oxidative stress response (Verneuil et al. 2005).

In *Bacillus subtilis*, OHRR protein is a homogeneous dimer belonging to a variety of antibiotic resistance (MarR) families. OhrR protein could regulate ohr genes express that encode thior peroxidase, and then the hydrogen peroxide is reduced to the corresponding alcohol. OHRR is resistant to hydrogen peroxide stress at the location of peptides at 15, through the oxidation of its unique cysteine residue. (Fuangthong and Helmann 2002). Cysteine is oxidized to sulfenic acid (RSH → RSOH) or to more oxidation state in oxidative stress.

Rex, the oxidation of an amino acid, was used to detect stress, and then the expression of cytochrome oxidase gene (cydAB) of *Bacillus subtilis* and streptomycin was controlled by the pool of NADH (Wang et al. 2008). This protein is a homogeneous dimer that contains two domains: the N-terminal domain is combined with the promoter region of the target gene (such as cydABCD), while the C-terminal identifies the ligand NADH. When the cell is at the stationary stage or the O_2 tension reduces, the NADH pool augments, causing it to combine with Rex. The Rex-NADH-DNA complex has been unstable, inhibiting the repression of gene that Rex controls.

5.5 Responses of Lactic Acid Bacteria to Cold Stress

5.5.1 Introduction

LABs play a significant role in the food and biotechnology industry. In the manufacture of foods such as yogurt, cheese (Crow et al. 2001), fermented meat, and vegetables and probiotics, as well as green chemistry applications (Axel et al. 2012), LABs are proverbially used as starters. Freeze-drying or lyophilization shows good

reproducibility in preserving bacteria. At supra-zero temperatures, it can be stored for a long time and at low cost as long as the water activity to values is reduced to less than 0.2. At the same time, low water activity to values assures that bacteria have little loss in viability and function. Adding protective agents into the bacterial suspension could keep bacteria stable in freeze-drying. Freeze-drying requires three measures: firstly, concentration frozen and cell suspension protected; secondly, ice removed through sublimation in initial drying; and finally, unfrozen water removed through desorption in secondary drying. In this chapter, a method was described to optimize the process parameters in freeze-drying of LAB, enabling bacteria to achieve the highest survival rates as well as greatest functional recovery.

Freezing is a commonly used technique in preserving, so the fast cooling rates are commonly adopted in food industry. Rapid freezing needs lower storage temperatures and usually results in the degeneration of vitality and acidification when thawing. Bacterial resistance to freezing relies on lots of parameters containing the bacterial species (Rault et al. 2007), protective additives, freezing rate, and final storage temperature. LABs are usually preserved through freeze-drying; however, lower temperatures could harm cells and cause devitalization. Over the years, attempts to improve the vitality of LAB during freeze-drying have been the focus of freeze-drying study.

By comparison, cold shock cannot lead to such clearly defined cell damage. Bacterial species exposed to lower temperatures always happens under different conditions. Food-related bacteria, for example, LAB, are specially and often encountered by lower temperatures in the process of food circulation. This situation that bacteria are exposed to low temperature happens more and more frequently in current few decades because refrigerator is invented to keep food and bacteria in cold storage. It's found that lots of bacterial species encountered by cold shock are able to temporarily induce arrays of specific proteins called cold-induced proteins (CIPs) (Wouters et al. 2001), as well as repress other synthesized proteins during actively growing or being exposed to other stress state, for instance, heat shock. This reaction is presumed to help cells overcome the physiological stress of cold shock. The downside effect of being encountered by cold stress is mostly due to the physical effect of lower temperature on cell structure and enzyme reaction. The cold shock reaction is a typical manifestation when the exponential growth medium is changed from the most suitable growth temperature to a lower temperature. In most bacteria, such as *Bacillus subtilis*, temperature reduction leads to the cessation of transient cell growth, during which common protein synthesis is severely suppressed. Nevertheless, these conditions touch off the synthesis of CIPs. Ultimately, this protein synthesis is reduced, and the cells become adapted to low temperature and growth recovery (Jones et al. 1987). The effects of cold shock can be observed at multiple levels: (1) membrane fluidity decreases, affecting membrane-related functions, such as active transport and protein secretion; (2) stable RNA and DNA secondary structure, resulting in reduced mRNA translation and transcriptional efficiency; (3) slow or inefficient folding of some proteins; and (4) in low temperature, the ribosomes have good adaptability under cold conditions (Phadtare 2004). In

addition, cold shock greatly disrupts the metabolism of bacteria cells; it is said that *L.casei* had a good network between metabolic regulation and cold reaction (Beaufils et al. 2007).

The reaction to freezing pressure is often passive, leading to a decrease in survivability and metabolic activity associated with low temperature damage (Guchte et al. 2002). By comparison, cold but positive temperature results in two adaptive responses. First, a subset of CIPs called cold-shock protein (CSP) was synthesized. The second is the change of membrane fatty acid composition, such as increasing the content of unsaturated and circulating fatty acids and allowing the membrane fluidity (Phadtare 2004). Transient physiological modifications of cell proteome patterns and cell membrane properties are generated by these adaptive responses, making bacterial cells better able to face farther pressure.

Application of a slow cooling rate allows for a repeatable freeze protocol, but the biological response of LAB needs characteristics during the freezing process. It is vital to know the basis of cold stress response of molecular mechanisms to improve the selection, storage, and performance of existing industrial stains.

5.5.2 Regulatory Mechanisms in the Cold Responses of Lactic Acid Bacteria

5.5.2.1 Cold-Stress Proteins

During cold stress, the most strongly induced proteins will include a family of CSP proteins. CSP proteins share high degree of sequence identity (45%) founded in many Gram-positive and Gram-negative bacteria (Phadtare 2004). CSPs proteins have been well known as transcriptional and translational regulators. They also act as molecular chaperones because they nonspecifically bind single-stranded nucleic acids and destabilize their secondary structures at low temperature (Gualerzi et al. 2003; Zeeb et al. 2006). CSP proteins are refered to establish a “new” cell balance in cold environment. However, the cell viability of several LABs increased after being sustain to a cold shock prior for freezing. That is to say, cold shock initiates the freezing tolerance, which also called cryotolerance.

The CspA-like protein and CSPs, which are from *Psychrobacter sp.B6*, have a high sequence homology (43%) with the Y-box factors, which are a family of eukaryotic nucleic acid-binding proteins. In these proteins, the domain involved in the nucleic acid binding is referred to as the cold-shock domain. This domain preferentially binds to the so-called Y-box, a nucleotide sequence element found in the promoter region of mammalian major histocompatibility complex class II genes. The Y-box could be characterized by a high converted sequence ATTGG (Phadtare 2004). This sequence was shown to exist in the promoter regions of at least two cold-shock genes, hns, encoding the nucleoid protein H-NS, and gyrA, encoding a subunit of DNA gyrase. It has suggested that CspA binds to the ATTGG element in

the promoter region of gyrA in *E. coli* (Panoff et al. 1998). It has been shown that the CspB can bind to the single-stranded DNA that contains the ATTGG element as well as the complementary CCAAT sequence (Panoff et al. 1998). Therefore, the result also suggested that CspA and CspB could act as transcriptional enhancers to cold-shock genes by recognizing the putative ATTGG sequence (Phadtare 2004).

The CSPs, which in a cell could be detected by the stability of the proteins. The CSPs of *B. subtilis* undergo rapidly folding and unfolding transitions and exhibit low conformational stability in solution. Certainly, in vitro conditions, CSPs also are rapidly degraded by proteases but are protected against proteolysis by binding to RNA.

The CSP genes of *B.subtilis* have been deleted, which induces compensatory effects of the remaining CSPs (Graumann and Marahiel 1997), and a similar response is suggested for *L. lactis* (Wouters et al. 2000). Interestingly, multiple deletion analysis showed that at least one functional CSP is required for cell viability in *B. subtilis*, indicating that CSPs play an important role not only during cold-shock adaptation but also during active growth under physiologic temperatures (Graumann and Marahiel 1997). The research found that most familiar LAB genomes have several homologous copies of *csp* genes. Nevertheless, a large part of them did not make an intensive study. It was found that the chromosome of *L. lactis* MG1363 had two pairs of cold-induced csp genes (cspA-cspB and cspC-cspD), the cspE gene and the putative cold-shock gene cspD2. *L. lactis* IL 1403 transformed from 30 to 15 °C, entering into a state of cold shock, meanwhile showing ability of ten times induction of cspB-mediated galactosidase activity (Wouters et al. 2000). It is reported that cspA of *E.coli* also expressed the activity of cold induction, simultaneously instantaneous induction happening at the level of mRNA and transcription (Goldenberg et al. 1996; Fang et al. 1997).

It is reported that *L. plantarum* strain NC8 has detected genes of TcspL, cspC, and cspP. At the same time, the overexpression of each CSP gene had different phenotypic effects on *Lactobacillus* plant (Derzelle et al. 2002, 2003). It is worth noting that *L. plantarum* cells have a large amounts of cspC transcript during the period of early exponential growth, and the excess expression of cspC improves the growth adaptation at optimum temperature (Derzelle et al. 2003). In science, CSP protein has an important influence on the process of fermentation, which could perform both at low or optimum temperature. Comparing with cold induction, the characteristic of csp gene that noncold induced of L. plantarum and L. Lactis contain a longer 5-UTR.The difference between them is that the transcripts are highly unstable, and the expression level of csp gene between *L. plantarum* and *L.lactis* is consistent (Wouters et al. 1999).

5.5.2.2 Membrane Integrity

The resistance of bacteria to freezing or frozen storage depends on many factors, for instance, species of bacteria, growing conditions, CSP protein's production, etc. The adaptability of *Lactobacillus* to cold stress is different from bacterial strain and

is related to pressure conditions. Nevertheless, in addition to inducing a specific set of proteins, key responses also included major changes in the composition of membrane fatty acids.

After describing successive physical events, we found that the cell membrane is the main target of damage. The barrier properties of cytoplasmic membrane are essential to the energy transduction system of *Lactobacillus* cells and rely on the physical state of lipid bilayer. As a matter of fact, it was affected by outside temperature. Exactly, it suggests that normal cellular function requires a membrane lipid layer to be in a state of liquid crystal at physiological temperature. At lower temperatures, the lipid bilayer experiences a reversible change that the state of fatty acid chains varies from disordered to ordered array. Therefore, there is an inverse relationship between the ratio of unsaturated fatty acid to saturated fatty acid (U/S) and the growth temperature (Suutari and Laakso 1992). In addition, some certain fatty acids are of great importance to stress response (Li et al. 2009). Fernandez Murga et al. observed a phenomenon that C16:0 and C18:2 fatty acids of *Lactobacillus acidophilus* growth at 25 °C are increased. The concentration of C18:1 fatty acid increased in the condition of at low temperature in *Lactobacillus plantarum* and at acidic pH in *Streptococcus thermophilus*, under osmotic stress in *Lactococcus lactis*. What's similar is that in C18:1, some decline happened under the condition of freezing in *Lactic streptococci* and spray-drying in *L. acidophilus* (Brennan et al. 1986). A high content of cycC19:0 is beneficial to the cryotolerance in *L. bulgaricus*, *L. helveticus*, *and L. acidophilus* (Gomez et al. 2000). The modulation of membrane lipids among high ratio of CFA/UFA and the concurrent rigidification cause *L. lactis* TOMSC161 incapable of resisting freeze-drying and storage stress (Velly et al. 2015). In addition, it seems that the membrane fluidity is measured directly by fluorescence anisotropy that is a fast and simple tool to determine the optimal fermentation time, making it possible to acquire antifreeze stem cells.

It is well-known that cold shock is unfavorable to the harmful effects of membrane fluidity on other non-biological stress. This feature was observed in *Oenococcus oeni*, a wine starter (Chu-Ky et al. 2005). The effects of the combination of cold, acid, and ethanol on membrane physical state and *O. oeni* survival were analyzed. Ethanol and acid shock induced membrane sclerosis, which was associated with total cell death. By contrast, *O. oeni* cells restored its ability to survive when suffering first cold shock (8 °C) and then ethanol and acid shock (Chu-Ky et al. 2005). These results indicate that the combination of cold, acid, and ethanol shocks has positive short-term effects on the membrane fluidity and viability of the *O. oeni*.

5.5.2.3 Freezing and Cryoprotection

Cold stress plays an important role in LAB cooling and freezing and is the primary cause of the loss of LAB activity. Additionally, stress from oxidation and/or hypertonic environments also occurs during the process of freezing and thawing (Stead and Park 2000).

When the bacterial cell mass is transferred from optimum temperature to low temperature, some bacterial strains can survive at extremely low temperatures, and this phenomenon is called freezing tolerance. The freeze-thaw challenge relies on temperature and the durability of cold pre-culture. Of course, it also depends on the initial concentration of bacterial cells. Cryopreservation of cell demands particular optimizations according to the type of microbe, and each type of cell has its own freezing solution. A growing number of studies are trying to develop ways to allow 100% preservation of the freezing–thawing of different cell samples; however, some microbes still do not apply to cryopreservation (Dumont et al. 2004).

During freezing–thawing cycles of LAB, many factors affect them, for instance, the composition and conditions of growth medium, growth stage, fermentation process, and low temperature (Streit et al. 2007; Siaterlis et al. 2009). Presently, a great number of methods have been put forward to maintain the quality of LAB and other probiotics (Panoff et al. 2000; Fonseca et al. 2003; Siaterlis et al. 2009) Among them, the use of cryoprotectant such as betaine, proline, glycerin, and trehalose is considered to be the most effective. These molecules improve cell preservation by reducing the amount of water and/or supporting vitrification and ultimately by preventing cells from forming large molecules (Dumont et al. 2004). It has been reported that *Lactobacillus reuteri* CICC6226 makes improvement of its membrane integrity and fluidity in the case of 10% trehalose or 10% remodeling of skimmed milk as a protectant in the freeze-drying process (Li et al. 2011). *L. sanfranciscensis* DSM20451 cells containing GSH than without glutathione showed higher resistance to freeze-drying, freezing–thawing, and cold stress induced by 4 °C cold treatment (Zhang et al. 2010). Cell contained GSH could maintain the integrated structure of the membrane when exposed to freezing–thawing treatment. Additionally, the cells that have GSH exhibited a high proportion of unsaturated fatty acids in the cell membrane during long-term cold treatment. The protective effect of GSH on cryo-damage of cell membrane partly results from the prevention of peroxidation and protection of fatty acids of the membrane. Intracellular accumulation of GSH enhanced the survival and biotechnological properties of *L. sanfranciscensis*, suggesting that the selection of GSH accumulation strains could improve the robustness of the initial yeast to the fermentation of sourdough.

In order to improve the freezing tolerance of lactic acid bacteria, it is essential to select an appropriate freezing and storage conditions and select resistant strains (Dumont et al. 2004; Monnet et al. 2003). Specific environmental conditions of fermentation, for example, pH, temperature, and centrifugation procedures, should be paid much more attention (Palmfeldt and Hahn-Hägerdal 2000; Beal et al. 2001; Shimrat 2005; Wang et al. 2005a; Streit et al. 2007). At the same time, it has been reported that the pH and temperature of fermentation were intensely affecting the freezing tolerance of *Lactobacillus acidophilus*. Diacetyl lactis can notably improve cell viability after continuous freezing and thawing (Lee 2004; Panoff et al. 1995; Wang et al. 2005b). Even so, it is innate feature that is crucial for bacterial strain (Fonseca et al. 2001).

The resistance of bacterial cells to freezing might also be improved by genetic engineering. For example, the overproduction of CSPs, CspB, and CspE has been shown to increase the survival of *L. lactis* after four freeze–thaw cycles of a ten- and fivefold factor, respectively (Wouters et al. 2000). Moreover, the overexpression of sHSPs in *L. plantarum* enables transformed cells to tolerate heat, solvent, and, importantly, cold stress (Fiocco et al. 2007).

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Chapter 6
Lactic Acid Bacteria-Based Food Fermentations

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https://doi.org/10.1007/978-981-13-1559-6_6

Abstract Food fermentation is essential for human being throughout the history as fermented foods enrich our diets. In particular, lactic acid bacteria play important roles in food fermentation, and they present us with foods in diverse aromas, tastes and textures. These edible microorganisms are found in pickled vegetables, sausages, cheeses, yogurts, sourdough breads, et al. The practice of lactic acid bacteria-based food fermentations happened accidentally at the beginning, but soon spread out for multiple benefits including preservation, safety, nutrition and flavor.

Keywords Fermented products · Biochemistry · Microbiology · Probiotic properties

Food fermentation is essential for human being throughout the history as fermented foods enrich our diets. In particular, lactic acid bacteria play important roles in food fermentation, and they present us with foods in diverse aromas, tastes and textures. These edible microorganisms are found in pickled vegetables, sausages, cheeses, yogurts, sourdough breads, et al. The practice of lactic acid bacteria-based food fermentations happened accidentally at the beginning, but soon spread out for multiple benefits including preservation, safety, nutrition and flavor.

6.1 Biodiversity of Fermented Products

For a very long time in human history, traditional food fermentations rely on spontaneous reactions of natural microbial communities associated with food. Although the dominate genera often vary between fermentations, practices such as backslopping and similar raw materials and associated microorganisms generate relative consistency even without any introduction of starter cultures. Under natural conditions, diversity in structure of raw materials, microbial community composition, and ability to form biofilm embody the microbial ecology dynamics under competitive environments. On the other hand, difference in processing methods of cheese can result in variations in microbial composition. Even under the circumstance of utilization of the same milk source and heat treatment for cheese-making variation in microbial diversity in cheese products occur due to variations in the pH, salt, moisture, and temperature during the cheese-making, and dramatic impacts on the aroma, flavor and texture of the cheese from the metabolism of cheese-associated microbes are observed.

It appears that in the modern food industry, with the application of pasteurization and pre-selected and pre-adapted genera, i.e. starter cultures, the rich microbial diversity of the native, regional microbial communities of natural fermented foods appear to be less important. However, as the demand of more diverse fermented foods arise from the consumers, it is essential to dig into the natural reserves of microorganisms of the natural fermented foods and understand the story behind them (Gibbons and Rinker 2015). Through the characterization of the diversity of microbial community and the dynamics during community formation, dominant

microbial groups can be cultured. Thus the in vitro communities can be reconstructed, and the comparison with the performance of the natural communities will facilitate the study of the mechanisms and principles of microbial community formation and dynamics (Wolfe et al. 2014). Food industry can benefit from in-depth characterization of the microbial consortia during fermentation for the improvement of lot-to-lot consistency, the identification of biomarkers for product quality or spoilage, and the manipulation of the fermentation conditions to improve grade and safety of the final products.

Ever since the notice of the roles of microorganism in food fermentation, humans have focused on the study of microorganisms in fermented foods, such as, controlling the composition of various microorganisms and regulating the production of metabolites, which could affect the texture, flavor, safety, and nutrition of fermented foods. Operation of production variables such as moisture, temperature, and salinity over diverse range of food materials could expand application offermented foods, including yogurt, cheese, wine, beer, sauerkraut, sourdough, kimchi, and salami (Table 6.1). The unique organoleptic sensations of these fermented foods usually rely on the characteristic microbial communities present in the fermented food.

As pointed out by Widder et al. (2016), "Despite a deep understanding of the composition of microorganisms microbial communities(MCs), we are still at the primary level in the dynamics and metabolism of MCs, which help predict and control MCs behavior". To reach our final goal of manipulation of the microbial communities for the design of fermented foods, challenges remain in the predictive mathematical model building with experimental data collection and method development (Widder et al. 2016).

6.2 Fermented Dairy Products

The science behind the transformation of milk into fermented dairy products is simple yet effective. First of all, the precious yet perishable nutrients in fresh milk need to be converted into more stable metabolites in the final products, which are achieved by multiple actions of lactic acid bacteria such as the partial consumption of valuable carbon and nitrogen source, lactose, which is essential to the microorganisms growth; the accumulation of lactic acid and the drop of pH; the removal of water and decrease in a_w; the production of metabolitesfor preventing the growth of pathogenic and spoilage microorganisms with acids and antibiotics. On the other hand, fermentation in milk generates diverse fermented dairy products with variations in appearance, aroma, taste, texture and nutrition, as a result of a series of biochemical reactions from carbohydrates, lipids and proteins.

Lactic acid bacteria are naturally used as starter culture in fermentation of milk, and the accumulation of lactic acid through fermentation coagulates the milk. Fermented milk, or yogurt, is popular all over the world, which is mainly fermented by lactic acid bacteria. Under certain circumstances, lactic acid bacteria together

Table 6.1 Major microbial group in different fermented food

Type of Food	Fermented Food	Major Ingredient	Main Microbial Groups	Reference
Dairy	Yogurt	Milk	Lactic acid bacteria	Sieuwert et al. (2008)
	Cheese	Milk, salt	Filamentous fungi; Yeast; Lactic acid bacteria; Acetic acid bacteria	Wolfe et al. (2014) and Montel et al.(2014)
	Tarag	Milk	Filamentous fungi; Yeast; Lactic acid bacteria; Acetic acid bacteria	Sun et al. (2014)
	Kefir	Milk	Yeast; Lactic acid bacteria; Acetic acid bacteria	Marsh et al. (2013)
Fruit	Wine	Pressed grapes	Yeast	Bokulich et al. (2014a) and Knight et al. (2015)
	Chocolate	Cacao pods	Filamentous fungi;Yeast Lactic acid bacteria	
	Coffee	Coffee cherries	Filamentous fungi; Yeast	Vilela et al. (2010)
Grains	Beer	Barley, hops, water	Yeast	Bokulich et al. (2012)
	Sake	Rice, water	Filamentous fungi; Yeast; Lactic acid bacteria; Acetic acid bacteria	Bokulich et al. (2014b) and Gibbons et al. (2012)
	Soy sauce, miso	Rice, water, soy beans	Filamentous fungi; Yeast; Lactic acid bacteria; Acetic acid bacteria	Bokulich et al. (2014b) and Gibbons et al. (2012)
	Makgeolli	Rice, water	Filamentous fungi; Lactic acid bacteria	Jung et al. (2012a, b)
	Sourdough	Wheat flour, water	Yeast; Lactic acid bacteria	Minervini et al. (2014)
Plants	Kimchi	Cabbage, spices, salt	Yeast; Lactic acid bacteria	Jung et al. (2011)
	Sauerkraut	Cabbage, salt	Lactic acid bacteria	Plengvidhya et al. (2007)
	Kombucha	Tea, sugar	Yeast; Lactic acid bacteria; Acetic acid bacteria	Marsh et al. (2014)
Seafood	Narezushi	Salt, vinegar, fish, rice	Lactic acid bacteria	Koyanagi et al. (2011)
	Kaburazushi	Vinegar, rice, fish	Lactic acid bacteria	Koyanagi et al. (2013)
Meat	Salami	Ground meat, salt	Filamentous fungi; Yeast; Lactic acid bacteria	Cocolin et al. (2011)

with certain yeasts or molds, could generate unique fermented dairy products. Co-fermentation of yeasts and lactic acid bacteria in milk generate fermented products containing alcohol such as kefir from Caucasian countries, koumiss from Russia and Siberia, mazun from Armeni. Viili, the scandinavian fermented milks fermented by lactic acid bacteria and mold, which have unique flavor and texture.

6.2.1 Biochemistry of Fermented Dairy Products

Fermented dairy products production is probably one of the oldest human practices for the preservation of milk, a perishable and nutritious food resource. Although the exact origin of fermented dairy products making is difficult to trace, the transformation not only extends the shelf-life significantly but also offers diversity through biochemical changes of the components in milk and action of microorganisms.

The technology applied for fermented milk production has been developed based on the physiological and biochemical characteristics of milk and the microorganisms involved. Although more innovative types of fermented dairy products are emerging into the market constantly, biochemistry remains essential for quality of a successful product in terms of flavor, texture, preservation and health-promoting properties.

6.2.1.1 Carbohydrate Metabolism

Milk is nutritious in nature with lactose, milk proteins, fat, vitamins and minerals. The fermentation of milk relies primarily on the presence of microorganisms which is able to metabolize lactose in the microbial community. Complex microbial communities are recorded in fermented foods, and the microorganisms which are unable to utilize lactose are often associated with lactic acid bacteria that can hydrolyze lactose. The symbiotic microbial community has been well recognized in fermented milk and kefir grain.

For a large amount of lactic acid bacteria applied to fermented dairy products such as lactobacilli, lactococci, leuconostocs and bifidobacteria, neither tricarboxylic acid cycle nor cytochrome system associated with electrons of NADH is the major pathway for energy. The energy obtained by these bacteria depends mainly on the phosphorylation of substrates and the ATPases of the cytoplasmic membrane. The pathways of carbohydrate metabolism include homofermentative and heterofermentative metabolism.

Since lactose is metabolized in the microbial cell, it is essential for lactose to pass the cell membrane to initiate the metabolic pathway. In certain LAB, PEP dependent phosphotransferase system (PTS) is required for the lactose transport, and lactose is phosphorylated by phosphoenolpyruvate (PEP) (Thompson 1987; Vos and Vanghan 1994). Then lactose-6-phosphate is hydrolyzed by bphosphogalactosidase (b-Pgal) into the galactose and glucose, which are catabolized via the Tagatose

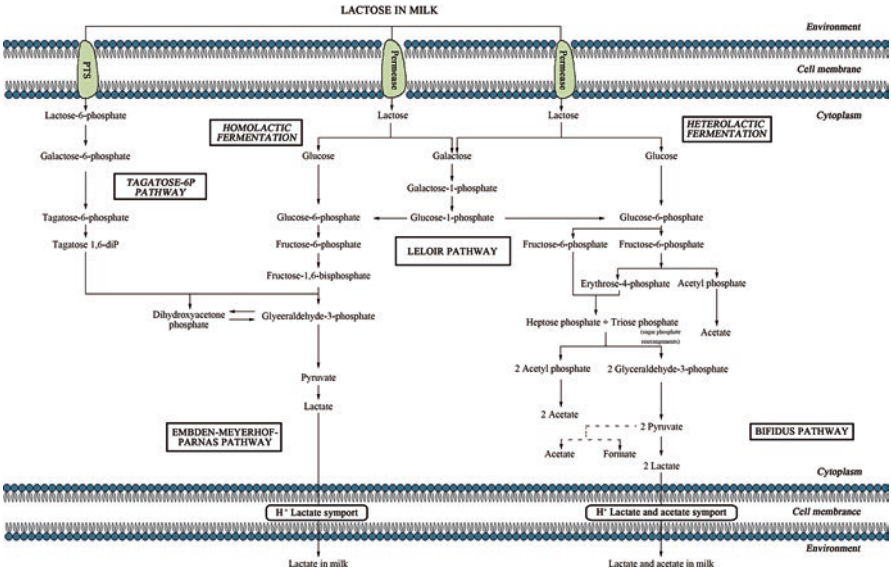

Fig. 6.1 Homolactic and heterolactic fermentation in lactose metabolism

and Emden–Meyerhof–Parnas pathways, respectively (Vos and Vanghan 1994; Marshall and Tamime 1997) and eventually turned into lactic acid (Fig. 6.1).

Recently, some comparative research based on KEGG database indicated that the operon for Tagatose-6P pathway is located in chromosomal of some strains like Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus paracasei, whereas Tagatose-6P pathway is plasmid-encoded in Lactococcus lactis (Wu et al. 2015). Firstly, lactose is absorbed by LAB via PTS, and then Lactose-6P is changed into galactose-6P and glucose under hydrolysis of phospho-β-galactosidase. Then glucose enter into EMP pathway and galactose-6P is converted into Tagatose-6P by galactose 6-phosphate isomerase. Tagatose-6P is eventually turned into glyceraldehyde 3-P for glycolysis. Actually, there are less enzymatic reactions involved in Tagatose-6P pathway for lactose metabolism while more complex reactions in Leloir pathway for galactose metabolism. However, enzymatic activity maybe limit the enzymatic kinetics of Leloir pathway, and less glucose-1P is be inclined to Leloir pathway for glycolysis via than Tagatose-6P pathway.

In LAB, some lactose is hydrolyzed into glucose and β-D-galactose by β-D-galactosidase, then the latter enter into extracellular medium. Those β-D-galactose return to the cytoplasmvia a lactose-galactose antiporter or galactose-specific permease, and is turned into α-D-galactose by galactose mutarotaseto involved in Leloir pathway. UDP-glucose and UDP-galactose from lactose are found to form via Leloir pathway and they are the main substrates for hetero-EPS production during milk fermentation. Although Leloir pathway also provides glucose-1P for glycolysis, the reactions involved may be limited by the related enzymatic activity. Therefore, Leloir pathway contributes mostly to EPS production while Tagatose-6P pathway prefer to providing more substrates for glycolysis.

Exopolysaccharide (EPS) produced by lactic acid bacteria (LAB) is long-chain, high molecular polymer during carbohydrate metabolism. In general, EPS is divided into capsular EPS attached to the cell wall of microorganism or ropy EPS secreted into the medium. Over the past decades, EPS has been reported to ameliorate the rheological characteristics, such as viscosity, texture, firmness and mouth feel, and to avoid syneresis during fermentation of dairy products (Bhaskaracharya and Shah 2000; Amatayakul et al. 2006a, b; Purwandari et al. 2007). Moreover, EPS could be a substitute for milk fat or chemically modified starches in fermented milk products. In addition, it is also reported that some EPSs have lots of physiological functionalities as antioxidative activities, anti-tumor effects and immunostimulative properties (Ruas-Madiedo et al. 2002; Broadbent et al. 2003; Fanning et al. 2012).

(a) Types of Exopolysaccharide

According to the chemical structure, EPS is generally classified into homopolysaccharides and hetropolysaccharides. Homopolysaccharides like glucans and fructans include one monosaccharide (glucose or fructose), while heteropolysaccharides are composed of repeating units of two or more types of monosaccharides which may contain galactose, glucose, fucose andrhamnose. What's more, homopolysaccharides could be produced by lactobacillus fermentum, lactobacillus mesenteroide and lactobacillus. sanfranciscensis.etc., whereas the producers of heteropolysaccharides are normally mesophilic and thermophilic LAB (Badel et al. 2011). Variation in composition, molecular mass, glycosidic linkage and charge determines different rheological properties of dairy products. For example, Backbone linkages of β (1 → 4) type, present in Lactococcus lactis subsp. cremoris B40, contribute to stiffer chains than α (1 → 4) or β (1 → 3) type (Behare et al. 2009). And α-linkages generally make the chains more flexible than β-linkages (Laws and Marshall 2001). Moreover, increase in molecular mass may be correlated with high viscosity (Looijesteijn et al. 2000).

(b) Biosynthesis of EPS

EPS biosynthesis is a complicated process via action of considerable numbers of enzymes and proteins. During fermentation of milk, EPS production has close association with galactose and lactose metabolism in thermophilic and mesophilic LAB, which are common starters including *S. thermophilus*, *Lc. Lactis* and *Lb. delbrueckii* (Wu et al. 2015). Four major steps involving EPS biosynthesis are nucleotide sugar synthesis, repeating unit synthesis, and polymerization and translocation of the repeating units. Firstly, galactose and lactose are transported from extracellular medium by the galactose-specific permease or lactose-galactose antiporter, and then converted into galactose-1-phosphate and glucose-6-phosphate by a series of kinase respectively for following enzymatic reactions about EPS biosynthesis. During the process, a key point linking the anabolic metabolism of EPS and the catabolic metabolism of sugar seems to be glucose-6-phosphate, which determines the direction of carbohydrate metabolism towards the formation of fructose-6-phosphate followed by glycolysis process and ATP formation or towards the synthesis of nucleotides sugar for EPS production. In addition, phosphoglutcomutase (PGM)

involving in transformation from glucose-6-phosphate towards glucose-1-phosphate could play a crucial part in the flux between the catabolic and anabolic pathways (Hugenholtz and Kleerebezem 1999). Afterwards, UDP–glucose pyrophosphorylase and dTDP–glucose pyrophosphorylase would convert glucose-1-phosphate, a branch process for the sugar nucleotides formation, into UDP–glucose and dTDP–glucose, respectively. Nucleotide sugar biosynthesis may be affected by carbon source (Ruas-Madiedo et al. 2002). Subsequently, Specific glycosyltransferases transfer various sugar nucleotides to form the repeating units connected by glycosidic bonds. And this kind of repeating unit is joined to undecaprenyl-phosphate-lipid carrier on the cytoplasmic membrane. The process of polymerization of the repeating units, and its translocation to extra cell, is not clear. But giving the high similarity between Gram-positive and Gram-negative organisms in the EPS polymerization and export, it is speculated that a 'flippase' may transfer the connected repeating units from the inner face of the membrane to the periplasmic face (Broadbent et al. 2003). Generally, growth physical conditions (temperature, PH and cultivation time) and medium composition (nitrogen and carbon sources) could affect EPS production and structure (Hassan 2008; Al-Dhaheri et al. 2017).

(c) Genetics of EPS Synthesis

So far, some research work on indentifying eps gene clusters have been carried out for identification of in EPS-producing LAB. The genes deciding polymers synthesis might be located in the plasmid of certain LAB, such as *Lactobacillus casei* and *Lactococcus lactis*, or on the chromosome, as in some thermophilic LAB (De Vuyst et al. 2001). Stingele et al. (1996) found the gene clusters encoding EPS synthesis in *S. thermophilus* Sfi6, identifying 13 genes (epsA to epsM) invovling EPS synthesis in a 14.52-kb region, regulated by a upstream promoter of eps A. To the present time, eps genes having been revealed in LAB are present in S. thermophilus NCFB2393, S. thermophilus Sfi39, S. thermophilus Sc136, *L. helveticus* NCC2745 and *Lc. lactis* NIZO B40 and so on. A high level of Hign similarity between these eps gene clusters of LAB reveals that these gene clusters function as a similar trend of regulation, determination of chain-length, the repeating unit biosynthesis, polymerization and translocation (Jolly and Stingele 2001).

(d) Strategies for improvement of EPS production

Due to the cellar energy limitations of LAB, EPS yield appears to be lower than other microbial polysaccharide. To increase EPS production and extend its application in food industry, strategies have been carried out via harnessing the genetic and metabolic capacity of LAB. One approach for overproduction of EPS bases on the biosynthesis level of EPS polymer, and by increasing activity of related glycosyltransferases involving this process. Increase of EPS production by similar overexpressions of epsD gene priming glycosyl transferase have been shown in *Lactobacillus helveticus*, S. thermophilus, and *Lactobacillus delbrueckii* subsp. Bulgaricus (Peant et al. 2005; Lamothe et al. 2002; Jolly and Stingele 2001). An alternative method to achieve a high EPS production is to increase copy numbers of the entire eps gene cluster on plasmid, but strain stability should be taken in consid-

eration. For example, *Lac. lactis* (MG 1363) obtained an increased yield via high heterologous expression of the entire eps gene cluster of *Streptococcus thermophilus* Sfi6 (Welman and Maddox 2003). Note that the application of such strains in the market should be exposed to regulatory controls and public acceptance. Of course, EPS biosynthesis depends partly on physical conditions which the strains grow. Certain studies showed that EPS production and growth obtained a higher level initially with the supplementation of medium by Skim milk powder (SMP) and whey protein concentrate (WPC) (Vaningelgem et al. 2004). Some other components in medium, as some amino acids, minerals and vitamins, could also influence the synthesis of EPS.

EPSs from lactic acid bacteria normally exhibit various composition and structure, thus offering special functional properties in health care products. Due to the low amount of EPS produced by LAB, the application to food-grade additives is still limited. So the controlling of fermentation conditions, with metabolic and genetic manipulation of EPS synthesis, might contribute to the higher levels of EPS or the construction of new polymers.

6.2.1.2 Protein Metabolism

Fresh milk is one of good protein resources for humans as it is rich in proteins. However, the lack of free amino acids in milk implies the importance of an efficient proteolytic activity for microorganisms associated with milk, which involves the serial break-down of protein into peptides and free amino acids. The degree of proteolysis in various fermented dairy products varies. In most of the commercial production of fermented milk, fermentation takes place within 4–6 h and proteolysis is limited, which results in relative less flavor compounds than in cheese that goes through aging.

Proteolysis is also important for the changes of milk matrix and texture of fermented dairy products. Entrapment of water is essential for fermented milk for avoidance of syneresis (wheying-off), and it is a general practice to add stabilizer to enhance the strength of protein matrix. However the demand of clean label lately calls for the investigation of lactic acid bacteria with the ability to develop high viscosity in fermented milk. On the other hand, in the aging of cheddar cheese, casein micelles fuse simultaneously with the development of the typical nutty and sulphury sensation of aged cheddar. (Deutsch. Microbiology and Biochemistry of Cheese and Fermented Milk - Springer[J]. Springer US.)

6.2.1.3 Lipid Metabolism

The mammalian milk contains various concentrations of lipids with a range between 2% and 50%, which is mainly related to the energy requirements of the species. In addition to the provision of energy, essential fatty acids and fat-soluble vitamins, lipids are also crucial for the flavor and textural properties of dairy products.

Therefore, the influence of fat reduction on the quality of the dairy products is one of the critical issues in the development of low-fat and non-fat products (Tamime et al. 2007).

Triacylglycerols account for nearly 97% of the total milk lipids, and the enzymatic hydrolysis of milk lipids eventually ends up with free fatty acids and glycerol. Since the lipases naturally present in milk are inactivated at pasteurization, generation of volatile fatty acids are mainly due to the lipolysis by starter cultures in commercial fermented dairy products. The importance of lipolysis to the flavor in fermentation of dairy products varies. For example, the contribution of lipid hydrolysis to the flavor and texture of fermented milk is less essential compared to the catabolism of carbohydrate and protein. However lipid hydrolysis contributes mostly to the flavor development of many cheese varieties. Free fatty acids have been connected to the cheesiness in Cheddar cheese, and the ratios of acetic acid to other fatty acids are believed to be an important factor of Cheddar flavor (Forss and Patton 1966; Forss 1979; Law et al. 1976).

6.2.2 Microbiology of Fermented Dairy Products

Most of the popular fermented dairy products have been traditionally made by spontaneous action of microorganisms, and carefully controlled microbial activities of starter cultures during the fermentation of dairy products are common practices today.

To date, dairy is the most studied environment and all kinds of cheeses were explored through amplicon-based high-throughput sequencing (HTS), responsible for curd fermentation monitoring (Ercolini et al. 2012) or cheese ripening (Fuka et al. 2013; De Filippis et al. 2016; De Pasquale et al. 2014b; O'Sullivan et al. 2015; Alessandria et al. 2016) and measuring the spatial microbes distribution of in different parts of the same cheese (O'Sullivan et al. 2015; De Filippis et al. 2016). In many cases, some researches highlighted possible relationships between microbial community structure/dynamics and physicochemical indicators, such as pH, water activity, salt concentration and temperature (Bessmeltseva et al. 2014; Lhomme et al. 2015a, b; Minervini et al. 2015; De Filippis et al. 2016). In otherresearches, the microbiota was influenced by raw material origin (Bokulich et al. 2014a; Rizzello et al. 2015) or quality (Dolci et al. 2014; Alessandria et al. 2016; O'Sullivan et al. 2015), as well as to formation of flavor-impact compounds (De Pasquale et al. 2014a, 2016; De Filippis et al. 2016). Moreover, food-related environments were found to develop a resident microbiota, beneficially involved in dairy (Calasso et al. 2016; Bokulich and Mills 2013a; Stellato et al. 2015), alcoholic (Bokulich et al. 2012, 2014b) and sourdough (Minervini et al. 2015) fermentations, although the presence of potential pathogenic microorganisms were also emphasized under some circumstances. (Bokulich et al. 2015; Stellato et al. 2015).

The lactic acid bacteria(LAB) are comprised of organisms which are Gram positive, non-sporulating, non-motile and primarily anaerobic, such as *Streptococcus thermophilus*, *Lactobacillus bulgaricus, lactococcus lactis, Lactobacillus casei Lactobacillus plantarum*, and *Lactobacillus helveticus*. Those bacteria produce lactic acid principally as the end product of sugar fermentation, while there are also many types of heterofermenting LAB producing ethanol, carbon dioxide, acetic acid and so on. LAB have been exploited in food fermentation for thousands of years due to their contribution to quality, flavor, texture and safety of the fermented products (Settanni and Corsetti 2008), thus enjoy a so-called "GRAS" (Generally Regarded as Safe) status.

6.2.2.1 Starter Cultures of Fermented Milks

A starter culture has been described by Hassan et al. (2001) as "any active microbial preparation intentionally added during product manufacture to initiate potential changes." The starter culture is one kind of the specific microbial culture used in the processing of fermented dairy products such as yogurt, butter and cheese, and is one key point affecting the whole production of milk fermentation and the stability of the product quality, the fermentation process is the consequence of the interaction of the starter and the microorganism in the milk (bacteria, mold and yeast) (Yu 2014). The starter cultures added during the production of fermented milk play two major roles: production of lactic acid and provision of flavor components. Although difference exists in the aroma, flavor, and texture of some regions, the fundamental manufacturing techniques are essentially consistent. The material is generally liquid or solid powder.

The mixed culture of two kinds of microbes grew well compared to they were separately cultured. This phenomenon was called symbiosis. The most well-known symbiotic growth in fermented foods is the application of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles*. They are all homofermentative lactic acid fermentation bacteria, and 0.7–0.8% L (+) lactic acid is produced in *Streptococcus thermophilus*, while *Lactobacillus delbrueckii* subsp. *bulgaricus* has strong acid resistance, and can produce more than 1.7% D (−) lactic acid. When two mixed fermentation, symbiotic effect makes lactic acid production faster than single strain, which not only shorten the fermentation time, but also reduce unnecessary microbial contamination due to long fermentation time, which is conducive to improving the economic benefits of dairy enterprises. Mixed fermentation with the two will greatly increase the rate of acid production, and to a certain extent, reduce the rate of post acidification, promote the production of flavor substances, and produce a lot of EPS (Alexander and Patrick 2001). The Streptococcus relies on the strong proteolytic activity of the Lactobacillus, and the Lactobacillus benefits from the carbon dioxide and formate from the Streptococcus. Yogurt is usually a product that is only fermented by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Its proportion is mostly 1:1 or 1:2, and bacilli should not take the advantage, otherwise the acidity is too strong. Many countries clearly stipulate that if the third kind

of bacteria is used at the same time in production, the products obtained after fermentation can only be called fermented milk. This kind of fermented milk mainly includes: Bifidobacterium fermented milk, Lactobacillus acidophilus fermented milk and *Lactobacillus casei* fermented milk. Mixed culture is often applied when Leuconostocs are involved in the fermentation. Although Leuconostocs is widely used in the dairy industry, they do not grow well in milk owing to the lack of proteolytic ability (Cogan and Jordan 1994). Therefore Leuconostocs act normally together with other strains with good proteolytic ability such as lactococci.

Kefir is a traditional fermented milk product popular in Poland, Turkey, and Northern Ireland, which depends on the fermentation of mixed microorganisms including non-lactose-fermenting yeasts (*Saccharomyces cerevisiae*) and lactose fermenting LAB (*Lac. lactis* subsp. *lactis* and *Lactobacillus kefir*). The use of mixed starter cultures can develop a number of nutritional health fermented milks, for instance, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are difficult to pass through the stomach in the active state to the intestines, but if the yogurt starter culture mixed with *Lactobacillus acidophilus* and *Bifidobacterium bacillus*, the dairy products have good health care effect.

When selecting the starter cultures, it is necessary to synthesize its viscosity, aroma production, protein hydrolysis, acid production and post acidification. Good starter should have the following characteristics: the ability to quickly generate acid; to give some yogurt flavor and texture; to produce extracellular polysaccharides and other substances to enhance the viscosity of yogurt; yogurt storage period with high survival rate; the various production conditions are easy to expand training; with activity and probiotic properties in healthy human gastrointestinal tract; in the 4–14 °C during the storage period after the acidification is relatively weak and so on (He et al. 2006) (Danielam et al. 2010).

In addition, good starter cultures also have many physiological functions: for example, to improve the utilization of calcium, phosphorus and iron in human body, provide many kinds of amino acid, vitamins and special enzymes, improve nutrition utilization, promote nutrient absorption and maintain body health including reduce the pH value of intestinal tract, promote intestinal peristalsis to prevent bacterial colonization, secret bacteriocins, inhibit the growth of pathogenic bacteria, regulate blood pressure and reduce cholesterol), activate the body's immune system to inhibit cell mutations to resist the occurrence of cancer, anti-aging effect (Beal et al. 1999; Wei et al. 2009; Hong et al. 2010).

At the start of the cheese-processing, some lactic acid bacteria, such as Lactococcus lactis and Streptococcus thermophilus, grow rapidly and generate lactic acid in milk. When it comes to aging, yeasts and moulds like *Penicillium camemberti* and *Debaryomyces hansenii* colonise the surfaces of cheese and utilize lactate (Callon et al. 2006). This contributes to the deacidification of the cheese surface, which benefits the bacteria that are less acid-tolerant to grow on the surfaces of cheese, such as *Arthobacter arilaitensis*, *Brevibacterium linens*. Bacteria, moulds and yeast have been chosen as cheese cultures in the industry for technological properties, such as pigmentation and aroma (Irlinger and Mounier 2009). However, it was found that those commercial cultures do not grow well on the sur-

faces of cheese. For example, *Brevibacterium linens*, the most widely used bacterium in aging cultures, was not found to cultivate successfully in cheese because it did not compete well with the native microflora present in the cheese-making environment. Actually, pure cultivation of strains does not result in a similar phenotype of microorganism relation (Irlinger and Mounier 2009). Therefore, it is necessary to pay attention to ecological adaptability of strains used in cheese culture due to their ability of adaptation in environmental conditions and to the interactions between microorganisms.

Lactococcus lactis is the principal component of dairy starter cultures used universally for producing numerous fermented dairy products including fermented milks like sour cream, buttermilk, and cheese both of commercial and artisanal origin. The main role of Lactococcus lactis in dairy starter culture systems is utilizing lactose to generate lactic acid at a sufficient rate and contributing to proteolysis in fermentation of milk (Wouters et al. 2002). Moreover, the flavor forming capability of Lactococcus lactis, especially *Lactococcus lactis* ssp. *cremoris*, has gained increased interest in dairy fermentation. Free amino acids act as precursors to the flavor compounds produced by microbial metabolism within the cheese environment (Yvon and Rijnen 2001). Amino acids converted into aroma compounds by aminotransferase, with the formation of an α-keto acid. It was reported that a raw-milk cheese and non-dairy Lactococcus lactis isolates produce the glutamate dehydrogenase(gdh), which converts glutamate to α-keto-gluturate (Tanous et al. 2002). Moreover, α-keto-gluturate is also the crucial compound that limits the rate in the formation of aroma compounds from amino acids. When the gene encoding gdh was cloned into a gdh- *Lactococcus lactis* strain, the more volatile flavor compounds were formed.

6.2.2.2 Starter Cultures of Cheese Manufacture

Cheese is one of the most popular fermented foods all over the world. With the advancement of understanding of the microorganisms associated with cheese-making, cheese-making has been customized in large plants. However the fact that there are over 2000 varieties of cheese existed indicates that cheese-making is an art instead of science. Traditional cheeses are biologically and biochemically dynamic matrixes with characteristic microbiota structure.

Starter cultures typically applied in cheese-making include lactococci, leuconostocs, lactobacilli, and Streptococcus. In some cases, starter cultures may also involve propionibacteria, brevibacteria, and mold (Penicillium) for a particular characteristic of cheese, such as the holes in Swiss cheese. Acidification is the essential step in cheese-making, which is often accomplished via the fermentation of lactic acid bacteria. Mesophilic cultures such as lactococci are typically used in cheese-making cooked below 40 °C, and thermophlic cultures are often used for cheeses that are cooked around 50 °C. The acids generated by lactic acid bacteria have multiple functions in cheese-making including the coagulation of milk, influ-

ence on the dissociation of colloidal calcium phosphate and textural properties of cheeses.

Fermented dairy products involved in many kinds of fermentation agent, we take Leuconostosis as an example to illustrate. Leuconostus is one of the most important starter cultures among the fermented dairy products. Leuconostoc is a type of Gram-positive bacterium that produces no spores and grows both aerobically and anaerobically. The best suitable growth temperature of most strains of Leuconostoc is 20–30 °C, and the fermentation type is heterogeneous lactic acid fermentation.

Since Leuconostoc can metabolize and produce appropriate amounts of CO_2 gas and various flavor substances, it takes an important part in the fermentation of dairy products (Hemme and Foucaud 2004). Leuconostoc is often used in the fermentation of butter, fermented milk and cheese. The CO_2 generated by Leuconostoc helps to soften the cheese and give it a good mouthfeel. *Leuconostoc mesenteroides* and Leuconostoc can perform specific citrate metabolism, producing diacetyl and aromatics, contributing to the formation of dairy flavor (Vedamuthu 1994), and can also be fermented as non-fermentative strains with other lactic acid bacteria. Therefore, breeding of Leuconostoc species with excellent traits will greatly promote the diversification of the types of fermented dairy products and improve the sensory effect and functional effect of the products.

Studies have shown that Leuconostuswas always involved in the early or late expansion of certain cheese varieties. For soft-matured cheese, the curd structure has high toughness and is not easy to collapse. The CO_2 gas generated from the metabolism of Leuconostoc can be used to form perforations in the cheese. It was found that, like Norwegian cheese in the Dutch Gouda-type, seven of the nine cheese products had perforation which were formed by Leuconostoc (Hemme and Foucaud 2004). For pressed ripened Dutch cheeses, such as Edam cheese, Gouda cheese or other saltwater-infused cheese varieties, tiny, fine holes are caused mainly by the CO_2 gas produced by Leuconostoc. For such cheese products, should try to choose a moderate amount of CO_2 generation strains, in order to avoid excessive gas production caused by excessive mesh phenomenon. In cheese production, when using a concentrated cell suspension of *Leuconostoc mesenteroides subtilisin* as a starter, it is often possible to obtain higher cell numbers in the product. In addition, Leuconostoc mesenteric cells, existing in the milk or coming from the environment, may also be used to produce a sufficient number of eyelet structures in the cheese. However, when pasteurized raw milk or high-hygienic raw milk (which means that there is a very small number of Leuconostocs naturally present in raw milk) is needed to process the cheese, it is necessary to add Leuconostas to raise the cheese quality.

Leuconostus used in dairy processing, producing the main metabolites including acetic acid, diacetyl, and ethanol, t will contribute to the formation of flavor products. Leuconostas further converts diacetyl to odorless acetoin and 2,3-butanediol. In practice, once the flavor material has been formed, these unfavorable methods of bioconversion can be reduced by cold-treating the product, such as fermented milk. Cooked cheese products are often stored at higher temperature (10~13 ° C), mainly

because this temperature range can be a greater degree of inhibition of diacetyl degradation. In addition, the increase in oxygen content will contribute to the formation of flavor compounds in the product. In cheese production, lowering pH, decreasing water activity, and increasing salinization all affect acetaminophen activity of *Leuconostoc mesenteroides* (Liu et al. 1997).

According to the actual situation, there are not some "so-called non-fermentative lactic acid bacteria" strains involved in the fermentation in the natural microbial flora of fresh milk. Therefore, many different attempts have been made to apply Leuconostoc to other types of cheese products after compounded with other non-leavening lactobacilli (eg, certain species of Lactobacillus). *Leuconostoc mesenteroides* are commonly used in thefermentation of butter, cream or fresh cheese, but their presence is hardly detectable in other cheese products, even though the presence of small quantities of living cells occasionally affects the quality of the cheese may also be negligible, which provides space for the action of *L. mesenteroides* subsp. *mesenteroides* or *L. mesenteroides* subsp. *dextranicum* (Cogan et al. 1997). With the deepening of research, more and more *L. mesenteroides* subsp. *mesenteroides* or *L. mesenteroides* subsp. *dextranicum* species are used as the preferred strains for the fermentation and production of various cheese products. However, most strains show relatively low rates of growth or no growth in milk or cheese curd. This condition can be improved by adding lactic acid bacteria co-fermentation method. Although the mechanism of action of Leuconostoc in some functional foods is not yet clear, it has shown a very good momentum of development. Further research on Leuconostus in functional fermented foods (including prebiotics) will expand the range of its applications in functional foods.

The microbiota of cheese is one of the essential factors affecting the sensorial properties of cheese. Therefore, the understanding of the microbial dynamics during cheese-making and aging is critical in prediction and control of the quality of cheese. Efforts have been made to investigate the evolution of microbial consortia in traditional and industrialized cheese of different varieties for the purpose of modulation of the quality of cheese and reduction of processing costs.

Various omics approaches have been applied to explore the dynamic microbial ecology and metabolism during cheese-making (Cocolin and Ercolini 2015), and metatranscriptomics has been used to study the behavior of inoculated strain cultures in model cheeses (Dugatbony et al. 2014; Lessard et al. 2014). Although the microbial communities of cheese are relatively predictable compared to those of the complex natural environments, the close relationship between the microbial community and quality of cheese indicates the importance of a better knowledge of the dynamics of microbial assembly.

Although the application of starter cultures are common in modern cheese-making, it is widely accepted that non-starter lactic acid bacteria are crucial in the flavor generation during cheese ripening, which is affected by the manufacture and aging conditions. During the cheese-making, carbohydrate metabolism occupies an important position. On the other hand, protein and lipid metabolism catch up during the cheese aging.

6.3 Fermented Cereal Products

6.3.1 Introduction

Cereal grains are of great significance because they provide various dietary nutrients for animal and human. Cereals and cereal-based products are recognized as one of the most important sources of carbohydrates, proteins, fiber and selected micronutrients for people worldwide (Đorđević et al. 2010). Although the production is huge, the lower content of protein, the deficiency of certain essential amino acids (lysine), the low availability of starch the presence of some anti-nutrients (phytic acid, tannins, alkaloid and polyphenols) and the coarse nature (Chavan et al. 1988), result in inferior or poor nutrient quality and sensory properties of cereals-based products. Lots of methods have been applied to ameliorating the nutritional qualities of cereals and cereals-based products (Blandino et al. 2003). Fermentation is considered to be the best one of all the processing technologies. It is well known that fermentation is one of the most important ways to preserve and provide abundance quantities of nutrient foods with a variety of sensory and texture properties.

Fermentation can be divided in many ways, for instance, lactic acid fermentations, alcoholic fermentations, acetic acid fermentations, alkaline fermentations. It is reported that vegetable protein meat produced by fermentation could substitute from legume and cereal mixtures (Steinkraus 1996). There are not very distinct lines between the classifications.

Lactic acid fermented cereal-based products are present all over the world. In Asia, there are fermented gruels, Chinese soy sauce, Chinese fermented bean curd, Japanese shoyu, Japanese miso, Indian idli, Indonesian tempe and ontjom, Philippine balao, Philippine puto, etc. In Africa, there are Egyptian kishk, Ethiopian enjera, Sudanese kisra, etc. In Europe, there are the most famous sourdough bread, Greek trahanas and so on. Throughout the fermentations of various cereal-based products, microbes are an integral part. This chapter concentrates on lactic acid bacteria in cereal-based products.

6.3.2 LAB in Cereal-Based Products

Cereal-based products represent a rich source of microbial diversity. Lactic acid bacteria (LAB) plays an essential part in fermentation, and is capable of accelerating acidification and steering the fermentation process subject to the produce of lactic acid and certain organic acids. The earliest fermentation was spontaneous and the quality of the final properties was relying on the microbiota came from the raw material naturally (Leroy and De Vuyst 2004). Microbe, including LAB, isolated from cereal-based fermented products showed in Table 6.2 (Singh et al. 2015).

Table 6.2 Major cereal-based products and the micro-organisms involved

Product	Main cereal	Microorganisms involved	Fermentative outcome	References
Kishk (Fugush)	Wheat (bulgur)	*Lactobacillus casei* and *Lactobacillus brevis*, *Lactobacillus plantarum*, Bacillus subtilis and yeasts, *S. cerevisiae*	Super preservation property, richer in B vitamins	Beuchat (1983); Blandino et al. (2003) and Tamime and McNulty (1999)
Idli	Black gram, Rice	*Lb. fermentum, Leuconostoc mesenteroides, Ent. faecalis, Pc. dextrinicus* and yeasts especially *Sacch. Debaryomyces hansenii, Pichia anomala and Trichosporon pullulans. Torulopsis holmii, Trichosporon pullulans and Torulopsis candida*	Batter leavening and flavor and gas formation, starch degradation; the accumulation of vitamin B and free amino acids, as well as the reduction of enzyme inhibitors, antinutrients and flatus sugars	Soni and Sandhu (1999); Nout et al. (2007); Nout (2009); Aidoo et al. (2006) and Farnworth (2005)
Mifen	Rice *(Oryza sativa*)	Pediococcus, Streptococcus, Leuconostoc, Lactobacillus, Enterococcus and Aerococcus spp. and the yeasts *S. cerevisiae, Candida rugosa*	Anti-microbial spoilage, and the modification of the starch granules facilitating their gelatinization during cooking. Better chewing qualities	Lu et al. (2005)
Ben-Saalga	Pearl millet (*Pennisetum glaucum*)	*Lactobacillus. plantarum, Lactobacillus. fermentum, and Pediococcus pentosaceus* and so on	Increased content of proteins and minerals , The reduction of antinutritional components (such as phytate)	Sifer et al. (2005); Lestienne et al. (2005); Mouquet et al. (2008); and Tou et al. (2006)
Jiu	Sorghum and a mixture of wheat, barley and peas (“daqu”)	Rhizopus, Mucor, Aspergillus spp., acetic acid bacteria, lactic acid bacteria, bacilli and yeasts, Saccharomyces, Candida, Hansenula spp.	Source of energy, stimulation of the digestive system	Zhang et al. (2007)

(continued)

Table 6.2 (continued)

Product	Main cereal	Microorganisms involved	Fermentative outcome	References
Mawe	Maize (*Zea mays*)	*Lactobacillus fermentum, Lactobacillus. curvatus, Lactobacillus. brevis, Lactobacillus. buchneri, Weissella confusa, Candida glabrata, Candida kefyr, Saccharomyces cerevisiae*	Formation of acidity and flavour, as well as enhancement of digestibility	Hounhouigan et al. (1994) and (1999)
Tchoukoutou	Sorghum	*S. cerevisiae* and lactic acid bacteria.	Better iron solubility in raw sorghum, i.e., by approx. 17% of total Fe in tchoukoutou protein.	Kayode et al. (2007) and Nout (1987)
Jnard	Finger millet (Eleusine coracana)	Rhizopus oryzae, Amylomyces rouxii, Endomycopsis fibuligera, Enterococcus faecalis, and so on		Hesseltine and Ray (1988) and Tamang et al. (1988)

6.3.3 Function of Lactic Acid Bacteria Communities in Cereal-Based Products

Many biochemical changes occur during cereal-based products fermentation to improve flavor, texture and digestibility, increase nutritive value, extend preservation, and in some cases, boost functional properties. The positive effects are owed to the accumulated metabolites, which are produced by LAB during fermentation, including organic acids, exopolysaccharides, enzymes, bacteriocin and so on (Gänzle 2014). The changing conditions during fermentation always provide optimum pH conditions for various enzymes, which play an indispensable role in all the metabolism. Sourdough represents far the most investigated cereal-based product, and the fermentations are generally dominated by microbiota, especially LAB (De Vuyst and Neysens 2005). Therefore, taking sourdough as an example to introduce biochemical actions during cereal-based fermentation and the final efforts on the quality of the products.

6.3.3.1 Carbohydrate Metabolism

(a) Starch

Starch occupies a central role in cereal. The degradation of starch at the dough stage provides dominant available carbohydrates and reducing sugars for fermenta-

tion. The degradation triggered by the metabolites produced by microorganism and the activating enzymes coming from cereal naturally, and they are interdependent during taking effect. Conversion of starch and maltodextrins showed in Fig. 6.2.

In Fig. 6.2a, α-amylase, β-amylase, and glucoamylase are exiting in dormant grains of wheat and rye, and during fermentation of sourdough they degrade starch to liberate maltodextrins, maltose, and glucose. Acidification modulates the pH lower than 4.5 and inhibits amylases activities, but glucoamylases activities are not affected under acidified condition. In sourdoughs key microorganism, containing *Lactobacillus. sanfranciscensis*, *Lactobacillus. reuteri* and *Lactobacillus. fermentum*, and other key organisms, including harbour maltose phosphorylase (MalP) and an 1,6-α-glucosidase (DexB) as sole glucan-hydrolysing enzymes (Gänzle and Follador 2012; Gänzle 2014). MalP, highly specific to maltose, phosphorylyses

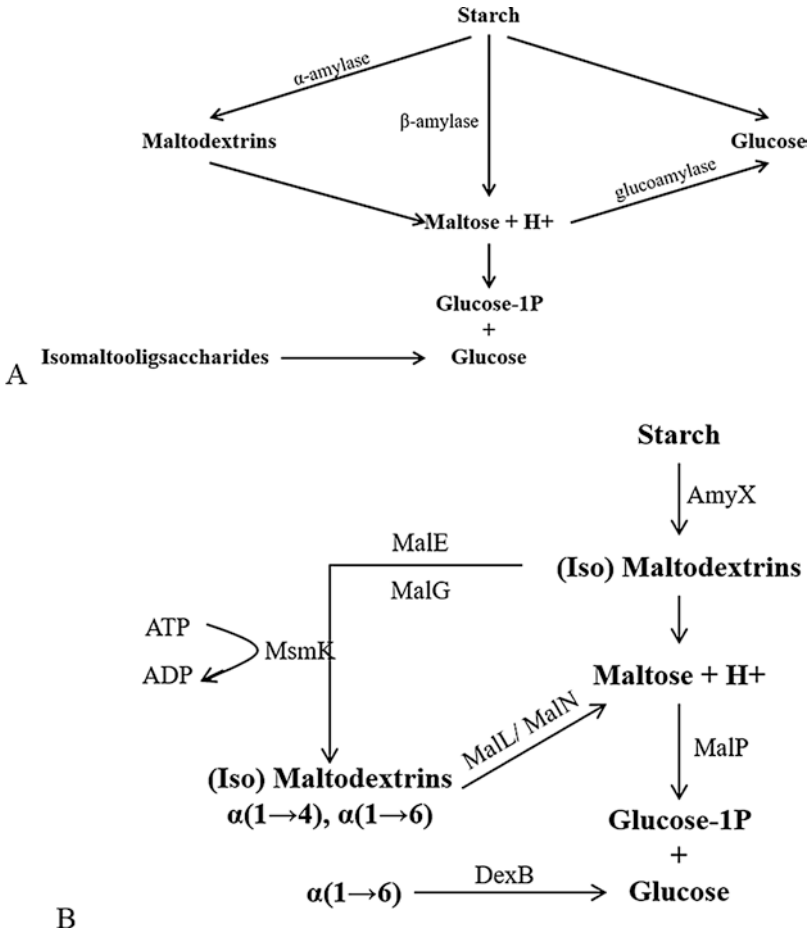

Fig. 6.2 Conversion of starch and maltodextrins metabolism in sourdough. (**a**) Metabolism of starch degradation to glucose in sourdough. (**b**) Starch degradation of cereal with low amylase activity

maltose to D-glucose and β-D-glucose 1-phosphate (Ehrmann and Vogel 1998). DexB hydrolyses α(1/6) linkages but not α (1/4) linkages in gluco-oligosaccharides (Gänzle 2014)

Figure 6.2b shows starch degradation of cereal, such as orghum, pearl millet, corn, and tubers (cassava, potatoes), with low amylase activity. In general, resting grains of C4 cereals do not have β-amylase activity (Taylor et al. 2006). Deficits in β-amylase activity is owing to low maltose concentrations in dough made from that kind of grains. During fermentation of these grains, starch degradation compeletly relies on exocellular amylases (AmyX) of LAB and amylolytic microorganisms. Extracellular amylase activity demonstrated in only few strains of lactobacilli (Gänzle and Follador 2012), but many strains are identified with amylolytic ability, including *L. plantarum*, *L. amylolyticus*, and *L. mannihotivorans* (Guyot and Morlon-Guyot 2001; Songré-Ouattara et al. 2008; Turpin et al. 2011; Gänzle and Follador 2012). The hydrolysisand transport of maltodextrin depends on enzymes and ATP-binding cassette transport system (Nakai et al. 2009), and many lactobacilli, such as *Lactobacillus. plantarum*, *Lactobacillus. acidophilus*, and *Lactobacillus. gasseri*, could provide a good source of enzymes (Gänzle and Follador 2012). MalN and MalL are endocellular glucosyl hydrolases, with the function of hydrolyse α(1/6)- and α(1/4)-glucosidic linkages in maltodextrins and isomaltodextrin (Nakai et al. 2009). MalP and DexB catalyse maltose phosphorolysis and isomaltodextrins hydrolysis, respectively. Remarkably, lactobacilli in fermentation of cereal contribute to the full complement of enzymes indispensable for starch hydrolysis, oligosaccharide transport, and hydrolysis (Moller et al. 2012; Gänzle and Follador 2012; Turpin et al. 2011).

(b) Arabinoxylans

According to researches, apart from major constituent starch, cereal grains always contain arabinoxylans, however, water soluble arabinoxylans only occupies a small fraction (Gebruers et al. 2010; Shewry et al. 2010). Benefiting from LAB, fermentation also provides optimum pH conditions for enzymolysis (Blandino et al. 2003). Xylanases, active in the pH 3.5–pH 5.5 and exist in raw cereal materials, contribute to solubilisation of arabinoxylans (Rasmussen et al. 2001; Gebruers et al. 2010). Researchers show that in rye sourdoughs the degradation of flour arabinoxylans increases the solution of high-molecular weight polysaccharides (Loponen et al. 2009). During sourdough fermentations, water-soluble arabinoxylans make contribution tohydration of dough andstability of foam, which bring the beneficial effects on texture properties of bread (Goesaert et al. 2005).

(c) Exopolysaccharide

Exopolysaccharide (EPS) is another important source of carbohydrate during cereal-based fermentation. The enzymolysis effects of glycansucrase trigger sucrose to form EPS during sourdough fermentation. LAB is indispensable to the formation of exopolysaccharide and take part in sucrose metabolism frequently. At least one exopolysaccharide producing LAB was observed in fermentation microbiota of tra-

ditional sourdoughs (Bounaix et al. 2009). Exopolysaccharide has significant beneficial effects on the viscoelastic properties of the dough, increasing dietary fibre content during fermentation, and slowing down starch retrogradation, and finally improving the volume, texture, and shelf life of bread. EPS is considered as improvers and potential to replace food additives, for instance hydrocolloids, used in bread making.

6.3.3.2 Protein Metabolism

(a) Proteolysis

Lactic acid bacteria (LAB) is a nutrient-demanding microorganism that requires exogenous amino acids and peptide-derived to growth (Hebert et al. 2004, 2008). In high protein culture media with low amino acid practicality, the growth relies on the metabolic products of proteolysis and peptidases. These enzymes work with peptides and amino acid transport systems in a coordinated manner and use proteins as a source of external amino acids (Savijoki et al. 2006). This proteolysis system is indispensable for the growth of bacterial because the accumulation of peptides and amino acids also plays an important part in formation of the organoleptic properties of fermented foods (Savijoki et al. 2006). Moreover, certain LAB strains release bioactive peptides that facilitate beyond basic nutrition (Hebert et al. 2008, 2010; Meisel 2004).

Microorganisms occupy a wide range of ecological niches in more diverse environments, for instance, fermented dairy products, sourdough and wine, to plant, soil and human gastrointestinal (GI) tracts. These organisms metabolic activity and cereal enzymes collaborate to degrade and depolymerize proteins during fermentation (Gänzle et al. 2008). During fermentation low molecular weight thiols accumulates along with acidification to trigger the solubilization of gluten proteins and finally result the vulnerability to enzymolysis (Jänsch et al. 2007; Thiele et al. 2004). Acidification applies the optimum pH to certain key enzymes, such as aspartic proteases (Bleukx et al. 1998; Brijs et al. 1999). During sourdough fermentation, endogenous proteinases are limited in proteolysis, so addition ofr fungal enzymes or malt is necessary for extensive protein degradation (Gänzle et al. 2008). The quality of bread is determined by polymeric wheat gluten proteins, which could play an important role to dough hydration and foam retention (Wieser 2007). During milk fermentation, casein occupied the mainly substrate, and other substrate is composed of proteolytic milk proteins (only 1–2% of the total milk protein) e and limited degradation of whey protein.(Khalid et al. 1991; Szwajkowska et al. 2011). The function of the proteolysis is that the fermented milk has a higher content of peptides and free amino acids than non-fermented milk, in particular histidine, valine, proline and serine (Matar et al. 2003). Lactic acid bacteria rely on its complicated proteolysis system for growth in milk to acquire all the necessary free amino acids. With strain-specific intracellular peptidases, lactobacilli contribute to the accumulation of amino acids during sourdough fermentation. A large number of LAB con-

sists of proteolytic enzymes that initially cut casein into polypeptide, peptidases (intracellular) that further degrade polypeptide into small peptides and amino acids, and some specific transport proteins that transport peptides and amino acids across the plasma membrane of cell (Kenny et al. 2003).

(b) Peptides and amino acids

Proteolysis directly contributes to release peptides and amino acids to cheese flavors. Much number of research on amino acid catabolism hasfocused on the fates of aromatic, sulfur and branched-chain amino acids by LAB because they play key roles in the development of aromatics. Two major routes to convert amino acids into flavor compounds: elimination reactions catalyzed by amino acid lyases and pathways initiated by amino acid aminotransferases. Amino acids are substrates transaminated, decarboxylated, dehydrogenated and reduced to produce various flavor compounds such as phenylacetic acid, phenethyl alcohol, methyl mercaptan, dimethyl disulfide, p-cresol, 3-methylbutyrate, 3-methylbutanal, 3-methylbutanol, 2-methylpropionate, 3-methyl-2-butanone, 2-methyl-1-propanal, 2-methylbutanal and 2-methylbutyrate.

In milk, LAB cannot be used to support significant growth on account of the low concentration of peptides and free amino acids. Therefore, LAB relies on their proteolytic system to release abundant amino acids and peptides to support growth in such environment. The assemblies of proteolytic systems of LAB consist of three fractions based on respective function: (1) proteinases that resolve caseins to peptides, (2) transport systems that transport the decomposition products across the plasma membrane, and (3) peptidases that degrade peptides (Kunji et al. 1996).

In LAB, peptidases are of capital importance part of the proteolytic system because they participate in the hydrolysis of peptides and provide essential amino acids (Christensen et al. 1999).

Metabolisms of peptides and amino acids are strongly linked to the sensory quality of bread, impact the formation of flavor compounds and their precursors, as well as bioactivators. During the fermentation of dairy products, consumer take notice of functional characteristics has prompted researchers to concentrate on bioactive substances formed by lactic acid bacteria (LAB). At present, milk proteins are regard as the primary source of bioactive peptides, and more and more bioactive peptides have been found in fermented dairy products and milk protein hydrolysates. (Clare et al. 2003; Silva and Malcata 2005; Korhonen and Pihlanto 2006). Strain- or species-specific microbe take active part in the conversion. For example, in rye malt sourdough fermentation, bioactive peptides accumulated by the lactobacilli with strain-specific peptidase activity (Hu et al. 2011). In sourdough fermentations, some peptides and amino acids metabolites were identified with function of antioxidant, antihypertensive, or cancer preventing (Gänzle 2014).

6.3.3.3 Conversion of Phenolic Compounds

Phenolic compounds in cereal are considered as main source of bitterness, and deter the absorption of starch and proteins (Dykes and Rooney 2006; Taylor et al. 2006). However, the beneficial effects of phenolic compounds cannot be neglected, for instance antioxidants (Ragaee et al. 2006; Dykes and Rooney 2006; Katina et al. 2007; Poutanen et al. 2009) and precursors for flavour compounds (Czerny and Schieberle 2002). However, the ecological role which phenolic compounds metabolism play is is not clear yet. Researchers speculate that the metabolism may apply metabolic energy by the liberate hexosides, and play the role of detoxification (Gänzle 2014). During fermentation the conversion and accumulation of phenolic compounds mainly depended on LAB, which harbor a full supplement of enzymes (Fig. 6.3).

Microbial enzymes catalyze the release of bound phenolic acids. During the releasing process, esters of ferulic acid were hydrolysed by feryloyl esterase, galloyl ester bonds of gallotannins were hydrolysed by tannase (tannin acyl hydrolase) (Iwamoto et al. 2008), the flavonoid aclycons were released from flavonoid hexosides by glycosyl hydrolases activity. In acid aseptic cereal fermentations, similar conversions are also reported, but the corresponding enzymes are not clear (Svensson et al. 2010; Hole et al. 2012). During cereal based products fermentations, phenolic acid decarboxylases and cinnamic acid reductases, which is strain specific, convert phenolic acids into other metabolites, and all these enzymes come from cereal-related lactobacilli (Svensson et al. 2010).

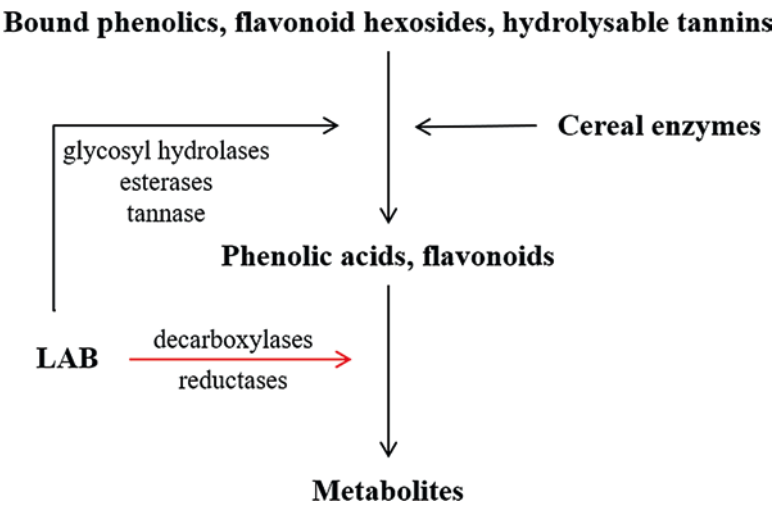

Fig. 6.3 Overview on conversion of phenolic compounds during sourdough fermentation

6.3.3.4 Conversion of Fatty Acids and Other Metabolites

Oxidation of lipid is in favor of generating aroma components, and influence dough rheology property during dough mixing. Several researches showed that hydroxy fatty acids have potential biological activities, for instance bacteriostatic activity (Black et al. 2013; Kobayashi et al. 1987). Metabolites of lactobacilli can promote lipid oxidation during sourdough fermentation, or play an effective role in antioxidative property. During fermentation Lactobacilli can hydrate oleic, linoleic, and linoleic acids to hydroxyl fatty acids, which is demonstrated to significantly extend bread's shelf-life without mould (Black et al. 2013).

Fermentation provides abundant metabolites more than the above introduction, such as optimum pH conditions for enzymolysis also release various minerals and vitamins (Blandino et al. 2003). Some minerals, for instance manganese, is an essential growth factor of LAB. Minerals and vitamins frequently enrich the nutritional value of bread. In fermentation, strains also produce antimicrobials such as bacteriocins to against the competition of other microbe (Leroy and De Vuyst 2004). Bacteriocin-producing LAB in fermentation is potential to combat microbial contamination and replace preservative used in bread making, finally extend shelf life of fermentative cereal-based productions.

6.4 Fermented Vegetable Products

6.4.1 History and Development of Fermented Vegetable

The origin of fermented vegetables can be dated back to the Shang Dynasty (3100 years ago). The first character to describe fermented vegetables was recorded in Book of Songs (Chen 2010). Chinese fermented vegetable technology was introduced to North Korea in 1300, and then was brought to Japan by Jian Zhen Before 1200. In seventeenth Century, fermented vegetables were introduced into Europe. Nowadays, fermented vegetables are wildly produced in different regions of the world and have various names in different countries (Table 6.3).

Kimchi was a traditional fermented vegetable in South Korea, which is made by the fermentation of vegetables, for instance, cabbage, radish, and cucumber with

Table 6.3 Fermented vegetables produced in the different regions of the world

Product name	Country	major ingredients
Sauerkraut	International	Cabbage, salt
Kimchi	Korea	Chinese cabbage, radish, salt
Paocai	China	Various vegetables, salt
Dakguadong	Thailand	Mustard leaf
Burong mustala	Philippines	Mustard
Almagro	Spain	Eggplant

various seasonings including salts, red pepper powder, garlic, leek, and ginger. Sauerkraut is manufactured with dry salting. Washed cabbage leaves are drained and put in a layer (Fleming et al. 1988). Salt is sprinkled over all leaves and then added another layer of cabbage, followed by sprinking salt again (Cocolin and Ercolini 2008). Paocai is a Chinese fermented vegetable using different fresh vegetable as raw material, and fermented in an anaerobic jar containing special Paocai brine (PB). PB, which is uninterruptedly propagated by back-slopping, contains stable level of lactic acid bacteria (LABs) and rich flavor and nutritional substances. In Sichuan, some areas still retain the customs that using PB to treat diarrhea, colds and other diseases (Cao et al. 2017).

Most spontaneous fermented vegetable products are highly dependent on the lactic acid bacteria (LABs) that are present in the raw materials, including *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and so on. LABs play a critical role in different fermentation vegetable, which contribute to the flavor, safety and probiotics properties of vegetables. (Chang and Chang 2010; Kim et al. 2013; Lee et al. 2011) This chapter addresses the contribution of LABs on fermentation vegetable products.

6.4.2 LAB Microflora in Fermented Vegetable

The beginning to elucidate the microbiology for fermented vegetable was made when Pederson revealed that *Leu. mesenteroides* initiated the sauerkraut fermentation (Pederson 1969). And then *Lb. brevis*, *P. pentosaceus* and *Lb. plantatum* were identified as dominated microbes of fermented vegetable at late fermentation stage. The techniques, such as microbial culture methods and the microbial classification methods, have limited the earlier studies on the microbiota of fermented vegetables. This inevitably results in misidentification and misinterpretation in studies of microbiota of fermented vegetables.

Over the years the development of molecular biology techniques is throwing new light on the studies of microbial communities of fermented vegetables. Several species of LABs other than the four species mentioned above have been identified in cabbage fermentations by microbial classification technology based on 16S rRNA gene, including *Lb. curvatus*, *Lb. sakei, Lactococcus lactis* subsp. lactis, and *Leu. fallax* (Björkroth et al. 2002; Martinez-Murcia and Collins 1990; Vogel et al. 1991).

In 1991, culture-independent method was firstly used to investigate the bacterial composition in environment samples (Ward et al. 1990). Substantial researches have been carried out to investigate the microbial communities of various fermented vegetable with culture-independent methods. Kim et al. demonstrated the microbial community composition of five commercial kimchi by 16S rRNA gene clone libraries. The result indicated that *Weissella koreensis* was dominated LAB in kimchi. Using culture-independent experiment revealed different microbiota from previous culture-dependent studies depended on phenotypic identification approachs (Kim and Chun 2005). Plengvidhya and colleagues (Plengvidhya et al. 2007) collected

686 isolates from four commercial sauerkrauts andtheir DNA fingerprints were analyzed. The results indicated that the species of LABs occurred in sauerkraut fermentations were more diverse than previously reported. New LABs were isolated from sauerkraut and identified as *Leuconostoccitreum*, *Leuconotoc argentinum*, *Lactobacillus. paraplantarum*, *Lactobacillus. coryniformis*, and *Weissella* sp.

With the development of high-thought sequencing technology, metagenomic approaches can provide more abundant and global information about gene content, metabolic potential, and annotate the function of microbial communities in fermented vegetables (Delong et al. 2006; Jung et al. 2013; Pérez-Díaz et al. 2017). Phylogenetic analysis based on 16S rRNA genes from the metagenome revealed that the kimchi microbiome was dominated by members of three genera: *Leuconostoc*, *Lactobacillus*, and *Weissella*. Surprisingly, a large amount of phage DNA sequences were identified from the fractions of cells, possibly suggesting that a high proportion of cells were infected by bacteriophages during kimchi fermentation. These results of culture-dependent and molecular studies altogether demonstrated complex and abundant LABs microflora in vegetables fermentation.

6.4.3 Function of LAB Microflora in Fermented Vegetable

6.4.3.1 Carbohydrate Metabolism

(a) Glucose and fructose

Fructose and glucose were considered as major free sugars in fermented vegetable. These free sugars play critical roles in the taste of fermented vegetables, because free sugars are not only sweeteners but also serve as important carbon sources for LABs to produce various metabolites. LABs involved in vegetable fermentations utilize sugars by two important pathways, including Homofermentation and Heterofermentation. Homofermentative *lactobacilli*, such as *Lactobacillus* and *Pediococcus*, convert hexoses by the glycolytic pathway to produce primarily lactic acid. Heterofermentative LABs, such as *Leu. mesenteroides*, *Lb. brevis*, metabolize glucose to lactic acid, acetic acid, ethanol and CO_2. Fructose can be fermented to the same products as a preferred electron acceptor to oxidize NADH back to NAD+. This characteristic result in that much of the fructose in a heterolactic acid fermentation is converted to mannitol. Mannitol is an important taste compound in fermented vegetables (Jung et al. 2012a; Xiong et al. 2014). Mannitol in fermented vegetable results in a refreshing taste, with noncariogenic properties, mannitol is considered to be a good replacement for sugars in diabetic foods (Wisselink et al. 2002).

(b) Sucrose

Sucrose also serve as a carbon source for LABs during vegetable fermentation. It is transported into cells through the enzymes or PTS system and hydrolyzed into glucose and fructose by sucrose hydrolase. Some *Lactococcus* utilize sucrose to

form 6-sucrose phosphate through phosphorylation. 6-sucrose phosphate is converted into 6-phosphate glucose and fructose via 6-phosphate hydrolase. PTS system and 6-sucrose phosphate hydrolase belong to inducible enzymes in lactic acid bacteria. In addition, glucan sucrase bind to cell wall of *Leuconostoc mesenteroides* could hydrolyse sucrose into monosaccharide for further fermentation.

6.4.3.2 Organic Acids

(a) Malic acid

LABs can produce lactic acid and carbon dioxide by decarboxylation of malic acid by malic enzyme. For some LABs, malic acid is metabolized to produce pyruvic acid and carbon dioxide. During the fermentation process, the reaction of malic acid to lactic acid mainly occurs in the initial stage of fermentation, even earlier than that of glucose metabolism. In addition, malic acid can also be converted to fumaric acid, and then form succinic acid. Through this pathway, the lactic acid accumulates rapidly, the pH decreases greatly, and the carbon dioxide is produced, which contributes to the formation of anaerobic environment. Therefore, this approach is very important for vegetable fermentation.

(b) Citric acid

According to the related genes of LABs and the growth environment, citric acid metabolism may produce different flavor products. The citric acid is decomposed into acetic acid and oxaloacetic acid by citrate lyase. Oxaloacetateis catalyzed into pyruvate by oxaloacetate decarboxylase. Pyruvate would be degraded to lactic acid, fumaric acid, acetic acid, diacetyl or acetoin. These compounds are important flavor compounds to improve the flavor quality of fermented vegetable.

6.4.3.3 Amino Acids

Amino acids play an important role in formation of taste compounds in fermented vegetables, which is accumulated during vegetable fermentation (Jeong et al. 2013; Sang et al. 2013). Proteases derived from raw materials and LABs microflora in fermented vegetables may contribute to the amino acid accumulation. LABs are obligate fermentative organisms, and they have incomplete TCA. In consequence, amino acid catabolic pathways in some cases are incomplete, divergent, or quite another thing to those described in model organisms. Transaminase and decarboxylase play an important role in amino acid metabolism of LABs (Fernández and Zúñiga 2006). It has been reported that LAB in fermented vegetables can degrade glutamic acid to γ-aminobutyric acid (GABA) by decarboxylase. GABA is considered to be a major inhibitory neurotransmitter in the central nervous system. In past studies, Lactobacillus species are proved to make great contribution to GABA producing during vegetable fermentation (Cho et al. 2007; Jeong et al. 2013; Kook and

Seo 2010; Seok et al. 2008). Therefore, Lactobacillus species including *Lactobacillus. sakei* and *Lactobacillus. buchneri* have been considered as kimchi starters for the production of GABA-enriched kimchi (Cho et al. 2007).

6.4.4 *Effects of Lactic Acid Bacteria on the Probiotic Properties of Fermented Vegetable*

LABs not only endow fermented vegetable unique organoleptic properties, but also probiotic properties of fermented vegetables. Fermented vegetable products that are mainly made by various LABs have been considered as functional foods due to the high concentrations of probiotic LABs (eg. *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus sakei* and *Lactobacillus casei*) and beneficial metabolites of LABs (e.g., antioxidants, extracellular polysaccharide, and GABAGABA). These LABs and metabolites of LABs have been considered to have potential health benefits, such as protective effects against heavy metal toxicity (Zhai et al. 2013, 2014, 2015a), antioxidant activities (Lee et al. 2005; Xing et al. 2015), alleviation of allergies (Ai et al. 2014, 2015; Han et al. 2012), inhibition the growth of Helicobacter pylori antidiabetic effect (Li et al. 2016), and antiobesity effect (Ji et al. 2012). Although a large amount of researches have shown the evidence to health benefits and potential probiotic effects of LABs isolated from fermented vegetables or in mouse experiments, the functions of fermented vegetables in humans were still unclear. Therefore, it is necessary to evaluate the probiotic effects and health benefits of fermented vegetables and their LABs on humans in further studies, which will promote fermented vegetables as a healthful food.

6.4.5 *Lactic Acid Bacteria and Safety of Fermented Vegetable*

6.4.5.1 Nitrite

Accumulation of nitrite is a recurring problem occurred during vegetable fermentations (Hashimoto 2001; Hou et al. 2013), especially in incompletely fermented vegetables (Chang et al. 2013). In the fermentation process, nitrate present in the vegetable's tissue is converted to nitrite by nitrate reductase. The content of nitrite increases to reach a maximum, and then decreases (Spoelstra 1985; 박건영 and 최홍식 1992). Nitrite is a precursor of N-nitroso compounds that can cause cancer and other health problems (Chan 2010). In consequence, decreasing the concentration of nitrite is important to preserve the safety of fermented vegetables (Xia et al. 2017).

A large amount of LABs isolated from fermented vegetables can degrade the nitrite (Oh et al. 2004). Substantial researches demonstrated that inoculation of LABs as starter cultures in vegetable fermentations are more adept at decreasing

nitrite concentration than spontaneous fermentation (Fan 1991; Oh et al. 2004; Yang 2004). Fei et al. compared nitrite degradation capacity of four *Lactobacillus* strains, *Lb. plantarum* DMDL 9010, which is isolated from Chinese pickle vegetable had a significantly higher nitrite degradation capacity than other strains (Fei et al. 2014). Xia et al. demonstrated that inoculation with *Lb. brevis* AR123 alone or combined with a commercial starter (mixed starter) is effective on removing the nitrites and enhancing the sensory properties of fermented vegetables (Xia et al. 2017). LAB could accelerate the degradation of nitrite significantly and shorten the fermentation time.

6.4.5.2 Biogenic Amine

Fermentation is an approach to preserve vegetables, because the organic acids produced by LABs fight against the growth of undesirable microbes. Nevertheless, high microbial counts may also release measurable toxic metabolites for instance biogenic amines (BAs) during the fermentation (Peñas et al. 2010). BAs is a group of natural compounds with biologically-active, which are present in fermented vegetables (Kalac et al. 2000). Kalacˇ et al. investigated the concentration of BAs in 121 sauerkraut samples and demonstrated that high content of tyramine and putrescine were present in these fermented vegetables. Kalacˇ and colleagues studied the changes of BAs concentrations during sauerkraut storage and revealed that the concentration of tyramine increased significantly during the storage, and was present at the highest contents, up to hundreds mg kg^{-1}. At the same time, high concentration of putrescine and cadaverine were detected during sauerkraut storage. In addition, if nitrite is contained in the fermentation system, biogenic amines and nitrite react to form nitrosamine, which is a strong carcinogen. Therefore, controlling of biogenic amine content is important for the safety of fermented vegetable.

Many methods can decrease concentration of BAs in fermented vegetables, such as temperature, pH, salt content, starters and storage environment (Bouchereau et al. 1999; Halász et al. 1994). Inoculating LABs as starters is one of the effective approaches to control the level of BAs in fermented vegetables. Pavel and colleagues revealed that the levels of tyramine, putrescine and cadaverine were significantly suppressed by *Lb. plantarum* during the vegetable fermentation (Kalač et al. 2000). Mohamed inoculated *Lactobacillus casei* at beginning of vegetable fermentation could effectively reduce the biogenic amine contents, such as putrescine, tyramine and histamine (Rabie et al. 2011). Furthermore the use of LABs to control the formation of biogenic amines, Peñas et al. demonstrated that both the individual and total biogenic amine concentrations in sauerkrauts stored at 4 °C for 3 months were below the upper limits reported in the literatures for fermented products. It is indicated that controlling fermentations and storage conditions also could alter the biogenic amine contents in sauerkraut (Peñas et al. 2010).

6.5 Functional Fermented Products

The inclusion of functional elements, particularly probiotics in fermented food is popular on these days with the demand of functional products from the consumers. The natural habitats of these probiotic strains are often non-dairy, including the mammalian gastrointestinal tract and other food resources such as Chinese paocai. The health benefits of bringing these probiotics into the fermented milk products can be classified into the following categories: (1) modification of the gastrointestinal (GI) tract activity and preservation of the microbial intestinal balance; (2) stimulation or modification of the host immune system.

More and more functional fermented products have been arising in various countries. However, the regulation and legislative frameworks vary significantly in different countries regarding functional foods. All over the world, the Japanese government is relatively supportive of the claims of health benefits of functional foods, as a large number of functional foods are being marketed under the FOSHU (Foods for Specialised Health Use) legislation annually. On the other hand, EU has more strict supervision over the health claims of functional foods.

Fermented foods are good vehicles for the delivery of functional LAB. The addition of LAB with the abilities of intestinal α-glucosidase inhibition and short-chain fatty acids production, and the utilization of prebiotics in symbiotic set yogurt significantly improve α-glucosidase inhibition (Muganga et al. 2015).

Fermented milk containing *Lactobacillus paracasei* subsp. *paracasei* CNCM I-1518 is shown to reduce the bacterial translocation in rats treated with carbon tetrachloride and indicated the protection in the intestinal barrier integrity (Sánchez et al. 2017).

Besides the well documented functional benefits of probiotic lactic acid bacteria, new functions such as alleviation of heavy metal toxicity has been reported including cadmium, copper et al. The investigation of protective effects against chronic cadmium toxicity of soymilk fermented with *Lactobacillus plantarum* CCFM8610 showed that oral administration of fermented soymilk was more effective than the treatment with non-fermented soymilk (Zhai et al. 2015a; b; Tian et al. 2015).

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Chapter 7
Lactic Acid Bacteria and Foodborne Pathogens

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Contents

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W. Chen, A. Narbad, *Lactic Acid Bacteria in Foodborne Hazards Reduction*,
https://doi.org/10.1007/978-981-13-1559-6_7

Abstract Foodborne pathogens are microorganisms which are capable of infecting humans via consumption of contaminated food or water, especially ready-to-eat products. The disease burden caused by foodborne pathogens has become a global public concern. Traditional technologies to reduce the foodborne pathogens includes preservative, refrigeration and pasteurization. In recent decades, probiotics showed emerging bacteriostatic and antifungal activity on different pathogens. This chapter discussed the trends of foodborne pathogens in the food-chain and the strategies for preventing and controlling food-borne diseases by lactic acid bacteria. The main mechanisms of the antagonism have been expounded, such as producing antibacterial substances, effect of competition and repulsion, adhesion barrier and immunomodulatory effects. Adjunctive application of prebiotics has also been described in this chapter, focusing on the physiological functions of promoting the growth of beneficial bacteria, adjusting the balance of intestinal flora and inhibiting the growth of pathogenic bacteria. In addition, the application of probiotics for graziery has also been depicted.

Keywords Probiotics · Foodborne pathogens · Prebiotics · Disease control

7.1 Introduction

Foodborne illness is a global public health challenge. Many environmental factors could result in foodborne illness, such as toxin, bacteria, parasites, viruses, metals, and prions (Thomas et al. 2013). According to a former literature review, 1415 species of infectious organism have been identified infective to human, including 217 viruses and prions, 538 bacteria and rickettsia, 307 fungi, 66 protozoa, and 287 helminths (Taylor et al. 2001). Over the last two decades, foodborne pathogens and diseases boomed, especially in the industrialized countries, and even caused political and media attention (Newell et al. 2010a). In order to reduce such disease burden, each procedure during the food chain has been monitored and encouraged to improve hygiene and food safety by incorporating structured approaches, such as HACCP principles (Hui et al. 2000). Certainly, many technologies have been proved to effectively reduce the foodborne pathogens, such as using preservative, refrigeration, and pasteurization. Some simple methods such as using uncontaminated food materials, cooking thoroughly, and keeping proper temperatures also largely prevent the production of foodborne pathogens (De Blackburn and McClure 2009). However, it is also necessary to learn deep about the interactions among pathogens and develop more effective prevention and control strategies. In this part, we attempt to discuss the trends of foodborne pathogens (including bacterial, viral, and parasitological pathogens) in the food chain and the strategy for preventing and controlling of foodborne diseases.

Table 7.1 Common foodborne pathogens and related disease (Amalaradjou and Bhunia 2013)

Pathogens	Clinical symptoms
Salmonella	Diarrhea, dysentery, vomiting, arthritis
Shigella	Arthritis, hemorrhagic uremic syndrome
Yersinia	Arthritis
Hepatitis A virus, *hepatitis E virus*	Hepatitis and jaundice
Campylobacter	Guillain–Barre syndrome, diarrhea, dysentery, vomiting, paralysis
Listeria, *Toxoplasma*	Miscarriage, stillbirth, central neural infection
Spongiform encephalopathy	Mad cow disease
Mycotoxin	Immune diseases
Seafood toxin	Allergy, paralysis
Clostridium botulinum	Paralysis
Vibrio, *Norovirus*, *Rotavirus*, *Entamoeba*, *Giardia*, *Isospora*, *Taenia*, *Bacillus*	Vomiting, diarrhea, dysentery

7.1.1 Foodborne Pathogens and Hazards

Foodborne pathogens are microorganisms as well as a number of parasites, which are capable of infecting humans via consumption of contaminated food or water, especially ready-to-eat products (Dwivedi and Jaykus 2011). The high prevalence of diseases caused by foodborne pathogens has become an important public health problem in the world. Although most symptoms are typically mild and self-limiting, some can cause disability and chronic sequelae and can even be fatal, especially in children. As a prevalent and serious threat, foodborne disease increases hospitalization rate and mortality in recent years (Thomas et al. 2015), over both industrialized and developing countries (Table 7.1) (De Blackburn and McClure 2009).

The four major foodborne pathogen types are bacteria, fungi, parasites, and viruses. Food can be used as a good vehicle to transport pathogens to appropriate sites and colonize in a new host. Bacteria are commonly found in soil and water; over 90% of confirmed foodborne human diseases and deaths reported by the Centers for Disease Control and Prevention are attributed to bacteria (Buzby et al. 1996). Since the 1990s, four major foodborne bacteria (*Salmonella*, *Campylobacter*, *E. coli*, and *L. monocytogenes*) have been the commanding indicators in most laboratory research and government surveillance agencies and, of course, covering the entire food industry (Scallan et al. 2011). For example, chicken is usually polluted with *Campylobacter* and *Salmonella,* uncooked eggs with *Salmonella enteritidis*, and ground beef with *E. coli* O157 (Newell et al. 2010b).

Salmonella are one of the leading causes of foodborne illness in humans. *Salmonella* are Gram-negative motile bacilli; there are more than 2450 serotypes included under two species *S. enterica* and *S. bongori*. *Salmonella* spp. widely colonize all the livestock species, including poultry, cattle, and pigs, which frequently

result in contaminated meat or other products (Stepanović et al. 2004). Some studies suggested that some *Salmonella* spp. also can attach to vegetables (Sant'Ana et al. 2012; Elizaquivel and Aznar 2008). In the European Union, *Campylobacter* spp. have been indicated as the most commonly reported food pathogen, causing acute bacterial food poisoning. *Campylobacter* usually existed in raw meats such as raw poultry meats, particularly in retail chicken products. The contamination rates have been reported even to 100% (Newell et al. 2010b; Zhao et al. 2001). *E. coli* is a normal commensal organism present in intestinal contents and feces, which is an excellent gut colonizer among many species. Based on their virulence factor and phenotypic traits, pathogenic intestinal *E. coli* strains have been classed into enteropathogenic, enteroinvasive, diffuse-adhering, enteroaggregative, and enterohemorrhagic *E. coli* strains. Most of current knowledge on foodborne *E. coli* infections is derived from Vero cytotoxin-producing *E. coli* (VTEC); among them *E. coli* O157 has been widely accepted as the cause of foodborne illness (Dhama et al. 2013). *Listeria monocytogenes* are important emerging foodborne pathogens in recent years, for it can survive under refrigeration conditions, low pH, and high salt concentration (Gandhi and Chikindas 2007). Listeriosis is a typical disease caused by *L. monocytogenes*, which is characterized by high mortality rate in affected individuals.

Fungi can cause spoilage of agricultural products before harvest, and sometimes also during postharvest conditions (storage), some of which can produce mycotoxins (mainly *Aspergillus*, *Penicillium*, and *Fusarium* species), enzymes, cellulose, volatiles, and potentially allergenic spores (Dwivedy et al. 2016). Aspergilli and penicillia are two common storage fungi, which produce aflatoxins, the most potent mycotoxins known (Abbaszadeh et al. 2014). Toxinogenic fungi are present widely in the animal and human foods. Moreover, commercial refrigeration conditions cannot prevent the growth of these fungi. Terrible harvesting and storage condition can also contribute to fungal growth and increase the production of mycotoxin, which has high thermal stability and multiple toxic effects, causing potential threat to human and animal health (van Walbeek et al. 1968).

Viruses are very small microorganisms, ranging from 15 to 400 nm. Viruses cause variable diseases in both plants and animals. Foodborne viruses are overall excreted in high numbers in human feces (Bauman et al. 2012). Only a few particles are needed for viruses to produce illness, while most of the viral particles are carried in the stools from infected persons. The host can be infected by viruses through different ways, a wide variety of foodborne viruses may be transmitted by the fecal–oral route (by infecting the cells lining the intestinal tract and are dispersed by shedding into the stool or through emesis), and it can be associated with diseases ranging from mild diarrhea to severe neural diseases. Nevertheless, the most frequently reported foodborne syndromes are gastroenteritis and hepatitis (Bosch et al. 2016). Different from foodborne bacterial infections, each group of viruses has its own typical host range and cell preference, because they are stable without the host and most of them are acid-resistant, but living cells are needed for their replication and migration to other organs (Koopmans and Duizer 2004).

From general food safety perspective, parasites have not been emphasized as other foodborne bacterial and viral infections, but the global food transportation

facilitated the wide spread of foodborne parasites, causing novel risks to animal and human health. Most bacterial and viral infections mainly caused acute gastroenteritis, while parasites may manifest with the chronic disease development (e.g., *Ascaris* spp., *Trypanosoma cruzi*, and *Trichuris trichiura*). However, acute disease may also occur (e.g., *cryptosporidiosis*) (Robertson et al. 2014; Robertson et al. 2013).

7.1.2 Strategy of Controlling Foodborne Pathogens

Foodborne hazards may come from physical, chemical, or microbiological origin. Strategies to prevent foodborne infections can be classified as preharvest and postharvest interventions. However, the increase in knowledge about the pathogens has led to the development of preharvest intervention strategies (Doyle and Erickson 2012). It is widely accepted that microbial foodborne hazards represent the greatest risk to consumers. Antibiotics are widely used in animal agriculture to control disease and improve animal growth rate and efficiency. Antibiotics exert its prophylactic effect through altering the microbiological ecology of the intestinal tract. While the spread of antibiotic resistance among microbial pathogens now threatens the long-term efficiency of current antibiotics, some pan-resistant bacterial pathogen strains are becoming more prevalent (Wittebole et al. 2014). Few therapies remain and it is urgent to find new ways. One of the alternatives is bacteriophage therapy. Bacteriophages (phages) are the most abundant microorganisms on earth (about 10^{31} particles) and existed in various foods (Brüssow and Kutter 2005). Since the very beginning, using phages to treat bacterial infection was controversial and without wide public support. Because of no controls and inconsistent results, many former studies were widely criticized. Bacteriophage therapy was essentially been rediscovered by modern medicine; they are widely used before the introduction of antibiotics, especially in Western countries (Wittebole et al. 2014). As natural preservatives, bacteriophages are suitable to decontaminate carcasses and other raw products, such as fresh fruits and vegetables, to extend the shelf life of perishable foods. They are also used to disinfect the contact surfaces of equipment (Garcia et al. 2008).

Some other antimicrobials, such as organic acids, essential oils, plant extracts, bacteriocins, and probiotics, have also been widely used in food safety approach that reducing the levels of pathogen loads in preharvest intervention (Soon et al. 2011). Organic acids are generally recognized as safe for chemical rinses (in the meat, fruit, and vegetable industry), such as acetic, lactic, and citric acids (Hassan et al. 2012). Organic acids reduce the pH of the food; in order to inhibit the growth of microorganisms, sometimes they are used in combination with oxidizing agents to enhance their antimicrobial efficacies (Sirsat et al. 2009). There are two primary mechanisms by which organic acids affect microbial activity: by acidification of the cytoplasm and accumulation of free acid anions. Organic acids have several advantages as antimicrobials, such as no limited acceptable daily intake dose, cheap, easy to manipulate, and slightly changing sensory of the product (Mani-López et al. 2012).

Many natural compounds in plants, herbs, and spices have been found to have some antimicrobial functions (Kotzekidou et al. 2008). The most well-known are "phytoalexins," which are small-molecule antibiotics (molecular weight < 500), including polyphenols, flavonoids, terpenoids, and glycosteroids, and they usually act synergistically (Karygianni et al. 2014). Plant extracts (extracted from clove, cinnamon, thyme, and oregano and their components) and essential oils are extensively used in significant amounts as flavor ingredients in the flavoring and perfume industries. Recently, they serve as a source of antimicrobial agents against the foodborne microorganisms, such as *Escherichia coli* O157:H7, *Salmonella enteritidis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* (Johny et al. 2010; Amalaradjou et al. 2010; Hyldgaard et al. 2012). Essential oils are not strictly oils, although they are poorly soluble in water and with pleasant odor and distinctive taste. Unlike antibiotics, essential oils have multifaceted antimicrobial effects, thus making it difficult for the bacteria to develop resistance (Karygianni et al. 2014). Bacteriocins are a group of low molecular mass peptides or proteins (no more than 60 amino acids). They are synthesized by bacterial ribosome, and released extracellularly, presenting antimicrobial effect on other bacteria, such as bacteriocin-producing bacteria, food spoilage bacteria, and other pathogens (Hoover and Steenson 2014). Majority of the studies focus on the production of bacteriocins from lactic acid bacteria (LABs), including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, and *Pediococcus*, while very few reports focused on the production of bacteriocins from *Bifidobacterium* (Cheikhyoussef et al. 2009). Bacteriocin is applied in the upstream procedure of veterinary, agriculture, and aquaculture, such as nisin and lacticin 3147, which have been incorporated into commercial prophylactic measures for foodborne bacteria. As an alternative choice to antibiotics, feeding bacteriocins have also showed good colonization in the gastrointestinal tract and reduced the endogenous pathogens (García et al. 2010).

Vaccine and antibody therapy also offer an effective strategy to reduce the foodborne illness. However, majority of the vaccines works only when the species being affected rarely establish the immune barrier for humans from the very beginning, such as animal source. Since there are some organism that could infect human, but not cause clinical symptoms in animal host, using vaccine to the animal would potentially reduce the risk of human diseases, and compared to the vaccination of human, it is more economical and convenient (Ghunaim and Desin 2015).

In addition, microbial contamination can also be controlled by physical methods, such as ionizing radiations, heating, and nonthermal methods (such as high hydrostatic pressure and oscillating magnetic fields). Irradiation is one of the most common physical methods of decontamination, by exposure to sufficient ionizing radiation to create positive and negative charges to destroy the indigenous flora, which prolongs shelf life of products during storage (Luksienė and Zukauskas 2009; Smith et al. 2004).

7.1.3 The Potential of Probiotics to Prevent Foodborne Pathogens

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2001). In recent years, probiotics are increasingly used for developing functional foods and dietary supplements. Probiotics exert beneficial effects in several ways, for example, probiotics can provide functional end products such as organic acids that can be utilized by the host, and they can also effectively compete with pathogen colonization to prevent disease. Besides, probiotics can stimulate host immune system and energy metabolism level and even influence human mood and cognitive abilities (Ouwehand et al. 2002; Rijkers et al. 2010).

The use of antibiotics in human and veterinary medicine to treat infectious diseases has led to the rise and spread of antibiotic resistance. Bacterial resistance to antibiotics has been a recognized reality, and the decreasing effectiveness of antibiotics and increasing numbers of antibiotic-resistant strains are spreading quickly within the past 20 years. Although antibiotics are used to target specific bacteria, the specificity can sometimes be too limited. The use of antibiotics in animal rations has also exacerbated the spread of resistance. Especially some broad-spectrum antibiotics can disrupt the intestinal microbial ecosystem and lead to the establishment of opportunistic pathogens (Newell et al. 2010a; Laxminarayan et al. 2013).

Due to the increased concern on the antibiotic resistance to pathogens, research has focused on the use of naturally occurring antimicrobials. Probiotics, prebiotics, and synbiotics (combination of prebiotics and probiotics) provide effective alternative to control foodborne pathogens in recent years. Competitive inhibition to the pathogenic strains is one of the advantages of probiotics, which decreases the pathogen development by producing antimicrobial substances such as bacteriocins, modified bile acids, and volatile fatty acids. The combination of antibacterial and immunomodulatory properties of probiotics has also opened a novel perspective. Administration of probiotics expressing antimicrobial peptides (AMPs) exerts significant effect on patients suffering from severe bacterial infections, particularly by antibiotic-resistant bacteria, and the use of immunomodulatory AMPs could eliminate these microorganisms by activating the host's own immune response (Mandal et al. 2014; Silva et al. 2011).

7.2 Probiotics to Control Foodborne Pathogens

The interaction between human and microbes established since the moment of all species appeared. Varieties of pathogenic microorganisms found in food caused major public health concerns, while amounts of beneficial probiotic organisms also existed in foods, and they have evolved with humans throughout history (Bourdichon et al. 2012). Probiotics can be used alone or with other organisms to positively shape the host's overall microbiota (Klaenhammer et al. 2012). A variety of

probiotic lactic acid bacteria (LABs) and *Bifidobacterium* have the capacity of stimulating natural killer cells. LAB is defined as an organism that produces lactic acid through fermentation, such as *Lactobacillus*, *Lactococcus*, and others. Besides, LAB produces a multitude of metabolic by-products with health benefits. LAB can be found in the mouth, vagina, intestines, and mucosal surfaces, which can occupy the nutrient-rich site by resisting the growth of pathogen (Josephs-Spaulding et al. 2016). Accompanied with the globalization of the food market, antibiotic-resistant and multidrug-resistant organisms also increased; a more comprehensive monitoring of the pathogenic organisms in food is needed. Due to increased consumer preference for wholesome and safe food, probiotics offer an effective and alternative strategy to control foodborne illnesses. Probiotics are found to be effective among the many intervention strategies investigated; many studies have demonstrated the efficacy of either wild-type or recombinant probiotics against foodborne pathogens and in preventing foodborne infections. This section will discuss the various probiotic-based intervention strategies in controlling foodborne pathogens.

7.2.1 Probiotics to Control Bacterial Pathogens

Bacillus cereus is an important food pathogen isolated from food samples, such as raw milk and dairy or rice products. Diarrheal and emetic types of food poisoning are principally caused by *B. cereus* (Palsboll et al. 1997).

Diarrhea-type enterotoxins are usually derived from dairy products, and some are transmitted through cooked rice. Raw milk is one of the major sources of *E. coli O157*. The survival rate of *E. coli O157* in fermented dairy products is one of the key points when causing disease in humans. Studies have shown that *Bifidobacteria* can inhibit the growth of pathogenic bacteria by producing organic acids that reduce the pH of the environment (Ibrahim and Bezkorovainy 1993). However, some reports mention that pathogenic *E. coli O157* is resistant to low pH conditions, and there is no significant reduction in survival and mortality in low pH foods (Meng et al. 1994).

Cheikhyoussef et al. (2007) investigated four *Bifidobacterium* strains (*B. adolescentis*, *B. bifidum*, *B. infants*, and *B. longum*) against *Bacillus cereus*, and three *E. coli* strains (*E. coli TG1*, *E. coli DH5a*, and *E. coli AS 1.543*) found that the antibacterial activity of these *Bifidobacteria* is not only related to organic acids but also related to the production of bacteriocins or bacteriocin-like compounds, which suggested their potential use as antibacterial additive in dairy products, such as yogurt and cheese. In another study, these *Bifidobacteria* have also been found to have the antimicrobial activity toward *Salmonella* ssp. All strains showed different degrees of antagonistic activity to the indicator bacteria. Among them, the inhibition rates of *B. infantis* and *B. longum* were 96% and 92%, respectively. *Bifidobacterium* and yogurt-mixed cultures have enhanced antagonistic activity to *Salmonella* and have shown great commercial value in the dairy and biopharmaceutical industry (Cheikhyoussef et al. 2008).

Antimicrobial proteinaceous compounds such as bacteriocins or bacteriocin-like compounds are known for the ability of inhibiting harmful microorganisms. They

are activated in various fields such as food preservation, health care, and pharmaceutical applications. It has been reported that the inhibitory activity of these substances is strain-dependent. Microflora colonizing the intestine have been found to be determined by the ability of binding to epithelial cells of the intestinal surface. Some substances produced by *Bifidobacterium* strains can compete and inhibit causative organism from adhering to receptors on the epithelial cell of the gastrointestinal surfaces. It is well known that the *Bifidobacteria* have potential probiotic effects in the human ecosystem, and the antimicrobial peptides they produced can help clarify the exact mechanism by which *Bifidobacteria* can regulate the gut microbiota and achieve its probiotic function. *B. infantis* BCRC 14602 was found to produce a bacteriocin-like inhibitory substance (BLIS), which showed inhibitory activities against a wide range of bacteria (Cheikhyoussef et al. 2009). The following studies purified the term bifidin I from *B. infantis* BCRC 14602 and confirmed its molecular mass, gene sequence, immunity phenotype, and the genetic determinant for the biosynthesis of bifidin I. Bifidin I from *B. infantis* BCRC 14602 is the first report on a bacteriocin since it is the only previously reported bacteriocin purified to homogeneity from *B. bifidum* NCFB 1454 (Yildirim et al. 1999). Because of the inhibitory effect on both Gram-positive and Gram-negative bacteria, bifidin I showed important potential for the application in food preservation. In addition, the production of bifidin I was regulated by a small plasmid in *B. infantis* BCRC 14602, and without immunity to bifidin I (Cheikhyoussef et al. 2010).

The antagonistic activities of *Lactobacillus plantarum* ST-III against the adhesion of *E. coli* and *S. enteritis* to Caco-2 cells were compared with the well-investigated *L. rhamnosus* GG. ST-III showed significant protective effects on strain, dose, and strain–dose interaction for *E. coli* and *S. enteritis*, and the most effective way for *E. coli* inhibition through ST-III was exclusion, whereas competition was the most effective mechanism for LGG. Co-incubation of ST-III with pathogens and Caco-2 cells after 3 h showed dramatic decrease of Caco-2 cell injury. ST-III could protect Caco-2 cells by competing for specific receptors or though steric hindrance against pathogens.

Previous studies have demonstrated the protective effect of lactic acid bacterium (LAB) against enteroinvasive *E. coli* (EIEC) infection. Administration of fermented milk containing probiotic bacteria could prevent the infection of EIEC in mice by enhancing the intestinal mucosa immunity (Medici et al. 2005). In order to cure EIEC infection using the antagonistic action of probiotics and explore a new potential probiotics from Chinese food resources, 339 bacterial strains were isolated from fermented dough, pickles, salted meat, baby feces, and dairy products. Using the in vitro assays of adhesion to HT-29 cell lines and tolerance to gastrointestinal conditions (acid and bile), two isolates, *L. plantarum* CCFM 233 and *L. plantarum* CCFM 231, were shown not only to inhibit the growth of EIEC significantly but also to have high adhesion to HT-29 cell lines and good tolerance to low acid and high bile. The two strains could strongly antagonize the adhesion and invasion of EIEC to the HT-29 cell lines (Liu et al. 2013).

Helicobacter pylori is a spiral Gram-negative microorganism that causes chronic gastritis, peptic ulcer disease, and gastric cancer. At present, triple therapies that use combinations of a proton pump inhibitor plus antibiotics (clarithro-

mycin or amoxicillin) and metronidazole are typically used as treatment. However, problems associated with patient noncompliance and consequent relapse of *H. pylori* infections are common. Moreover, these drugs destroy the microenvironment in the stomach, leading to side effects. Several studies reported that lactobacilli have inhibitory effects on *H. pylori*. X. Chen et al. screened 38 *Lactobacillus* strains for anti-*H. pylori* activity using in vitro methods, including survivability under the simulated gastric conditions, agar plate diffusion, urease activity, coaggregation, autoaggregation, and hydrocarbon analysis. Two *Lactobacillus* strains (*L. plantarum* 18 and *L. gasseri* Chen) showed significant potential anti-*H. pylori* activity compared with the other strains (Chen et al. 2010). In the following studies, the antagonistic activities of *L. gasseri* Chen and *L. plantarum* 18 were assessed by agar plate diffusion assay and tests that determined the growth and urease activity of *H. pylori* cocultured with lactobacilli and the adherence of *H. pylori* to human gastric epithelial cells in the presence of lactobacilli. The results indicated that the two *Lactobacillus* strains could inhibit *H. pylori* adherence to human gastric epithelial cells, and the antagonistic activity of the cell-free supernatants against *H. pylori* depended on the pH and the presence of metabolites, such as organic acids and proteases (Fig. 7.1) (Chen et al. 2012).

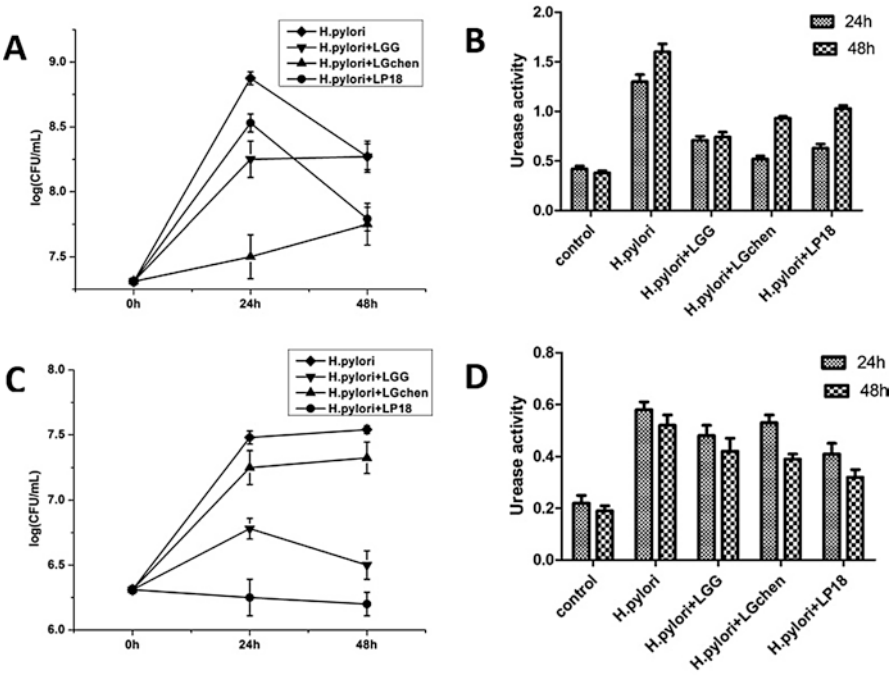

Fig. 7.1 (**a**) The live cell count of *H. pylori* SS1 in the coculture with lactobacilli; (**b**) The urease activity of *H. pylori* SS1 in the coculture with lactobacilli; (**c**) The live cell count of *H. pylori* SS1 in the coculture with the cell-free supernatant (CFS) of lactobacilli; (**d**) The urease activity of *H. pylori* SS1 in the coculture with the cell-free supernatant (CFS) of lactobacilli

Listeria monocytogenes has been recognized as a severe threat to sanitation, which affects pregnant women, babies, and people with weak immune systems. The US Department of Agriculture Food Safety and Inspection Service established a zero-tolerance limit on *L. monocytogenes* for ready-to-eat foods (Lewus et al. 1991). To improve the microbiological security of *L. monocytogenes* in food products, Gang Wang et al. isolated 285 lactic acid bacteria from fermented foods such as koumiss and pickles, and their antimicrobial properties were characterized. Among the strains, *Pediococcus acidilactici* P9 (a strain isolated from a local pickled vegetable) produced an antagonistic substance that inhibited *L. monocytogenes* and *Shigella*. It also showed an ability to protect HT-29 from invasion by *L. monocytogenes*, showing a potential for application in food products as a biopreservative (Figs. 7.2, and 7.3) (Wang et al. 2014a).

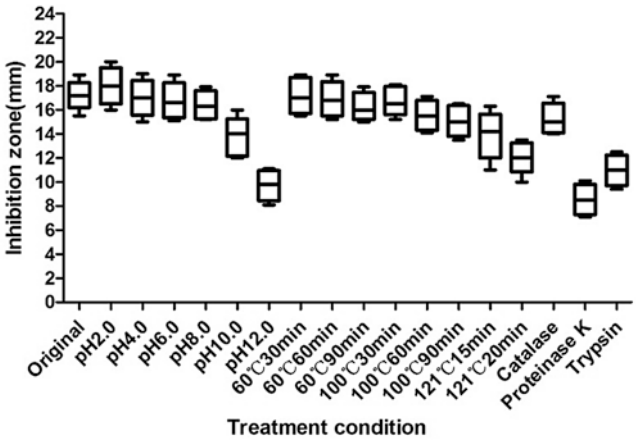

Fig. 7.2 Inhibition ability of antimicrobial substance produced by *P. acidilactici* P9 under different pH, temperatures, and enzyme treatments

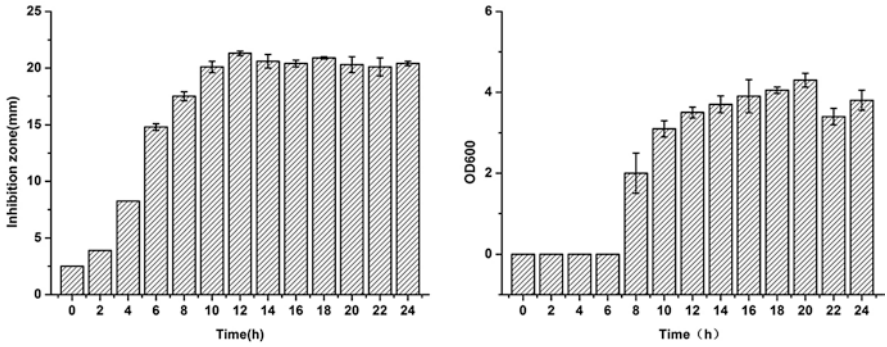

Fig. 7.3 Antimicrobial substance production during the growth of *P. acidilactici* P9 in MRS broth at 37 °C

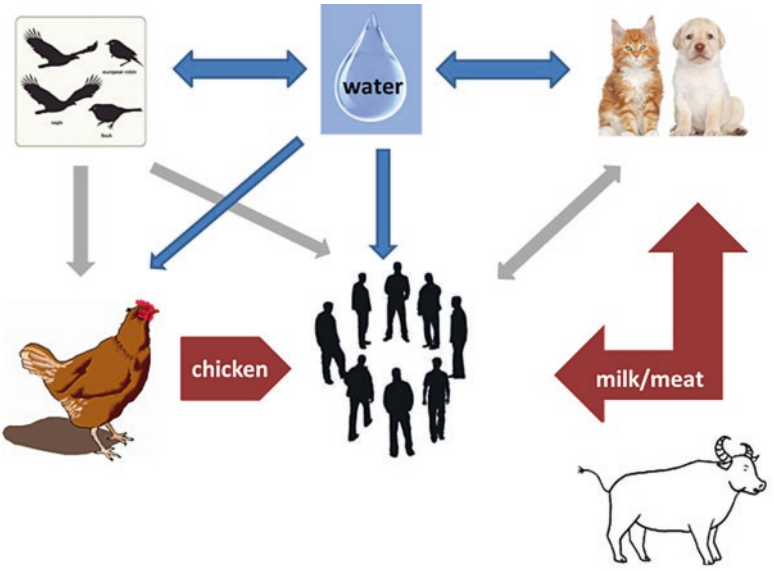

Fig. 7.4 Most important routes for human infection by *Campylobacter jejuni*

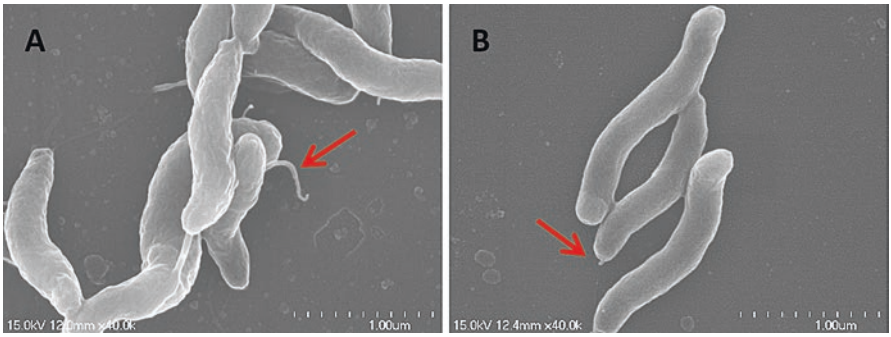

Fig. 7.5 Scanning electron microscopy of *C. jejuni* invasion. (**a**) Control group; (**b**) *Lactobacillus* intervention group

Campylobacter infections usually lead to watery or bloody diarrhea, accompanied with abdominal pain. In many countries, it has been identified as the leading cause of human enterocolitis (Figs. 7.4, and 7.5) (Dasti et al. 2010).

At present, antibiotics are commonly used as the treatment of diseases caused by *Campylobacter*. However, the increasing antibiotic resistance to *Campylobacter* strains suggests that novel approaches are necessary to be developed alternatively (Alfredson and Korolik 2007). Some studies have also evidenced the protective effect of lactobacilli in preventing *Campylobacter* infections. *Campylobacter jejuni* is now recognized as one of the main causes of bacterial foodborne disease in many

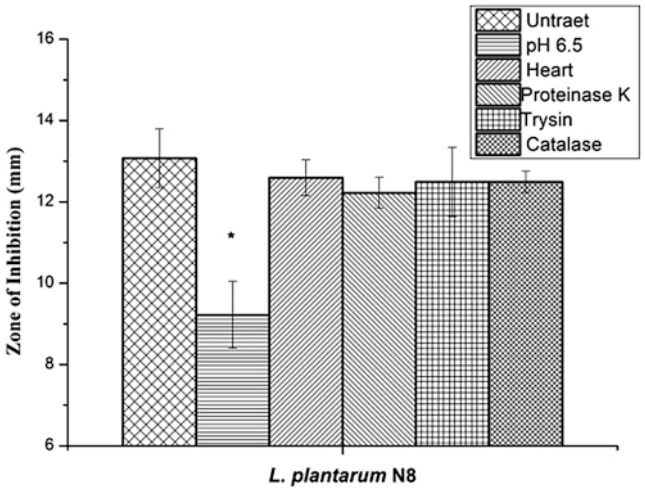

Fig. 7.6 Effect of different treatments on the antimicrobial activity of *L. plantarum* N8 CFS against *C. jejuni*

countries. Gang Wang et al. explored new potential probiotics of antimicrobial activity against *C. jejuni*; four adhesive *Lactobacillus* strains *L. plantarum* N8, N9, and ZL5 and *L. casei* ZL4 selected from 78 LABs were capable of exerting significant antagonistic activity against *C. jejuni* in vitro and promoted effective inhibition of adhesion and invasion of HT-29 cells by *C. jejuni*. The bactericidal capacity of these four *Lactobacillus* strains may probably be relative to the low pH and the production of metabolites, such as lactic acid and antibiotic-like substances (Fig. 7.6) (Wang et al. 2014b).

7.2.2 Probiotics to Control Fungus and Viral Pathogens

Probiotics are live nonpathogenic microorganisms which have antifungal activity and have been used to control fungus. A series of studies considered the use of metabolites from sourdough lactic acid bacteria such as phenyl lactic acid (Corsetti et al. 2000) to inhibit hazardous substance during bread storage (e.g., fungal contamination) (Gobbetti 2010). Organic acids and peptides from sourdough fermented (*L. plantarum* LB1 and *L. rossiae* LB5) wheat germ (SFWG) showed antifungal activity of *Penicillium roqueforti* DPPMAF1 (Rizzello et al. 2011). Grass silage containing *L. plantarum* strain (MiLAB 393) has been evaluated for its efficacy in controlling filamentous fungi and yeasts. Cyclic dipeptides cyclo(L-Phe–L-Pro) and cyclo(L-Phe–*trans*-4-OH-L-Pro) produced by lactic acid bacteria could inhibit fungus on grass silage storage (e.g., *Fusarium sporotrichioides*, *Aspergillus fumigatus*) (Strom et al. 2002). The studies of which against *Pichia anomala*, *Penicillium roqueforti*, and *Aspergillus fumigatus* have been reported about other lactic acid

bacteria also detected in grass silage which could generate antifungal compounds (Coda et al. 2010; Lavermicocca et al. 2010).

In the dairy industry, contamination with undesirable molds has been a serious and frequently disturbing problem that results in huge losses due to spoilage of cheese and other fermented foods incriminated by a variety of mycoflora such as *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, and *Mucor*. Some lactic acid bacterias have excellent features on the antifungal aspects, and the prospects of this exceptional trait as a potential food biopreservative have been reported (Dang et al. 2009; Guo et al. 2012). The antifungal activity of partial dairy propionibacteria (e.g., *Propionibacterium acidipropionici*, *P. jensenii*, *P. thoenii*) against food- and feedborne molds and yeasts was evaluated. The results showed that propionibacteria exhibited a pronounced species variation in antifungal activity on MRS agar and propionic acid which were the main antibacterial materials produced by propionibacteria (Lind et al. 2005). Fungal species such as *Penicillium* would make rice product decay, for example, rice cakes, because of its high moisture level and similar level of calories. The acetic and lactic acid produced by lactic acid bacteria such as *Leuconostoc citreum* and *Weissella confusa* were very effective in inhibiting the growth of the three fungal isolates, namely, *Clostridium* sp. YS1, *Neurospora* sp. YS3, and *Penicillium crustosum* YS2, and increase the shelf life of products, meaning that the lactic acid fermentation metabolites could be effective inhibitor to limit fungal growth in rice cakes (Baek et al. 2012). On bakery products, lactic acid bacteria also could be a novel food-grade antifungal agent such as *Lactobacillus amylovorus* DSM 19280 which has been shown to produce a broad spectrum of antifungal compounds active against common bread spoilage fungi and extended the shelf life of bread compared with some additive as calcium propionate (Baek et al. 2012).

Viral pathogens also can be controlled by probiotics. Viral gastroenteritis is an infection caused by a variety of viruses. The most common viruses that cause gastroenteritis are *Norovirus* and *Rotavirus*. Human *Rotavirus* which was inoculated in gnotobiotic pigs would make animals sick, while infected pigs fed with *L. acidophilus* and *L. reuteri* enhanced IFN-γ and IL-4 responses in serum and decreased the viral pathogen infection (Wen et al. 2009). *Norovirus* has been recognized as the major enteric virus responsible for 58% of foodborne illness in the world (Marshall and Bruggink 2011). Probiotic-fermented milk containing *L. casei* Shirota strain has been evaluated for its efficacy in controlling norovirus gastroenteritis in a health service facility. A total of 77 people were enrolled in the study. Intake of probiotic-fermented milk by the treatment group resulted in a reduction in the mean duration of fever after the onset of gastroenteritis (Nagata et al. 2011). BALB/c mice oral administration of a recombinant *Lactobacillus* strain expressing the hemagglutinin of the avian influenza virus H5N1 triggered both mucosal and systemic immune responses. With the increase of IL-4, anti-HA IgA and anti-HA IgG levels have the same changing trend (Wang et al. 2012).

Receptor mimetics has been used as a strategy to control foodborne pathogens. Many enteric pathogens recognize oligosaccharides expressed on host cells as receptors for toxins and adhesion factors. This interaction between the ligand and the receptor is essential for the initiation of infection process. This critical step in

the infection process can be adapted to develop intervention strategies. This involves the expression of molecular mimics of host oligosaccharides in probiotic bacteria to control enteric pathogens (Paton et al. 2010).

7.2.3 The Mechanism of Probiotics Inhibiting Pathogenic Bacteria

7.2.3.1 Probiotic Bacteria Produce Antibacterial Substances

Probiotics can inhibit the growth of a variety of pathogenic bacteria. The inhibitory effects of probiotics are connected with its antibacterial substances such as oxidation of hydrogen, lactic acid, acetic acid, bacteriocin, and so on. Whether Gram-positive or Gram-negative bacteria, bacteriocins as proteins produced by bacteria have an antiseptic effect for pathogenic bacteria along with the growth of probiotics. A large number of organic acids (e.g., lactic acid, acetic acid) associated with the growth of lactic acid bacteria are formed, and the pH of fermented liquid for lactic acid bacteria would be decreased. It is reported that the low pH has the obvious bacteriostatic effect for ferment liquid of lactic acid bacteria. Some scholars found *Lactobacillus* GG hinder the ability of *S. typhimurium* to intrude into Caco-2 cell when they adjusted the *Lactobacillus*-fermented liquid pH to neutral (Lehto and Salminen 1997). There was a possibility that the bacteriostatic effect produced by lactic acid bacteria was connected with antibacterial substances such as protein under low pH condition. Alakomi et al. (2000) thought the lactic acid produced by lactic acid bacteria could increase the permeability of pathogen; it would be beneficial to antimicrobial substance infiltrate into pathogenic bacteria cells and increase the sensitivity against pathogenic bacteria. Hydrogen peroxide produced by lactic acid bacteria in the bacteriostatic mechanism is not clear. When the hydrogen peroxide was formed, it would react with thiocyanate to generate some microbial toxic acid salt and then inhibit pathogenic bacteria.

7.2.3.2 Competition and Repulsion from Probiotics

Because lactic acid bacteria cannot express adhesion factor related with pathogen, the effects of inhibition from probiotics may be realized by receptor sites on placeholder pathogen cell. There were some reports that *L. casei* and *L. johnsonii* La1 could form specific binding with some sites of glycolipid which were also the cell adhesion site on the surface of pathogenic bacteria (Yamamoto et al. 1996). The construction of *Bifidobacterium* BL2929 attachment proteins which was similar to ETEC could inhibit ETEC to adhered cells, because it could compete with ETEC to combine with some sites of glycolipid such as asialo GM1 and GA1 (Fujiwara et al. 1997). In addition to adherence sites of glycolipid, lactic acid bacteria could secrete albuminoidal substances which could inhibit combination from pathogen to

adhesion receptor. Some scholar found that the ability of *L. crispatus* inhibition for pathogenic *Escherichia coli* was reduced when the surface protein had been removed. It was confirmed that the surface protein of lactic acid bacteria also inhibited the adhesion on pathogen (Horie et al. 2002).

7.2.3.3 Adhesion Barrier from Probiotics

The intestinal pathogenic bacteria can increase the permeability of the intestinal mucosa. Pathogenic bacteria and its macromolecular substances can enter into the intestinal mucosa and then destroy the mucous membrane barrier. Probiotics not only prevent and repair the damage to the intestinal mucosa but also weakened the injury from pathogen to host. It is reported that some *Lactobacillus* can stimulate the intestinal cells to secrete mucin, and the mucin can inhibit pathogen adhesion. The expression quantity of mucin MUC2 and MUC3 increased when *L. plantarum* 299 V was co-cultured with HT-29 cell, and the contact between ETEC and HT-29 was limited (Mack et al. 1999). *L. acidophilus* can hinder the damage from drug to the tight junction protein and play a great role on the protective efficacy. In addition, some lactic acid bacteria can also increase the membrane resistance across the monolayer cells and maintain the stability of the cytoskeleton and barrier function (Otte and Podolsky 2004).

7.2.3.4 Immunomodulatory Effects of Probiotics

There are two primary aspects on immunomodulatory effects of probiotics on the host: one is probiotics influence the nonspecific immune response. The probiotics would enhance the ability of mononuclear macrophages and polymorphonuclear leukocytes and stimulate the secretion of reactive oxygen, lysosomal enzyme, and monokine. In addition, lactic acid bacteria can induce specific immune response. There are many kinds of probiotics to enhance the host immunity, for example, through improving the expression quantity (e.g., IgA, IgM, IgG) to strengthen the humoral immune and promote the proliferation of T lymphocyte and B lymphocyte. Christensen et al. (2002) studied the adjustment of *Lactobacillus* on rat's dendritic cell surface markers and cytokines. The scientists found that the *Lactobacillus* increase the expression of the cell surface markers MHCII and B7-2 (CD86) which means that the *Lactobacillus* could stimulate dendritic cells to mature and produce IL-12. It was confirmed that *Lactobacillus* could enhance the host's immune responses. Animal experiments showed that the peripheral blood white cells and peritoneal macrophage phagocytosis activity had been enhanced by oral lactic acid bacteria in mice. It is reported that *B. lactis* HN019 could help people to significantly increase the number of $D40^+$ and $CD25^+$ T cells and natural killer cells in blood after taking *B. lactis* HN019 through clinical test (Gill et al. 2001). Volunteers were injected lower toxicity vaccines after oral *L. rhamnosus GG* 7 days, and the specificity of IgA antibodies in the blood content had been improved (Fang et al.

2000). As a potent inducer of TNF-α, probiotics can play a role of immune regulation on the body through stimulating the host to produce IL-6, IL-10, and IL-12 factors (Morita et al. 2002). But the immunoregulatory effects of probiotic mechanism are complex, and some immune pathways or the exact molecular mechanism is unclear. Future studies are essential to explore the immune regulation mechanism of probiotics.

7.2.4 Prebiotics

"A nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" defined as a prebiotic in 1995 (Gibson and Roberfroid 1994). But in 2007, it was defined as "a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host wellbeing and health" (Roberfroid 2007a). The chemical nature of prebiotic is oligosaccharides, but not all oligosaccharides have the prebiotic characteristics. Some parts of oligosaccharides which cannot be digested and absorbed by the human body called functional oligosaccharide accord with the prebiotic standard. There are some kinds of functional oligosaccharide that had been reported and applied.

7.2.4.1 Inulin

Inulin has been completely confirmed belonging to the functional oligosaccharide, and it is one of the most important prebiotics. Inulin, which is a kind of catenarian amylose, consisted of D-fructose through β(1 → 2) glycosidic bond. Glucose base is located at the end of inulin. Inulin molecule is mainly composed of 30–50 fructose residues, and its degree of polymerization is between 2 and 70 commonly. It has the high content in Jerusalem artichoke and dahlia. It is reported that inulin has the probiotic role. Some scholars found that inulin was beneficial to host through irritating the development of salutary fecal coliform bacteria and inhibiting the growth of pathogens and detrimental microbes (Tárrega et al. 2010).

7.2.4.2 Galacto-oligosaccharides (GOS)

Galacto-oligosaccharides are another functional oligosaccharide which meet a criterion of prebiotics completely besides inulin. Galacto-oligosaccharides are a natural attribute of functional oligosaccharide, and its molecular structure is galactose or glucose molecules connected with 1 to 7 galactosyls. It is reported that galacto-oligosaccharides ingested in the body could not be digested and absorbed by the host. No hydrolase on oral cavity and intestinal tract was the main reason for that,

and galacto-oligosaccharides would be utilized by beneficial bacterium such as *Lactobacillus acidophilus* in the intestinal tract (Rycroft et al. 2001).

7.2.4.3 Fructo-oligosaccharides (FOS)

Fructo-oligosaccharides can regulate the intestinal flora as the peptic carbohydrates and improve the resistance of intestinal infection on the host as food additives (Rodenburg et al. 2008). Fructo-oligosaccharides are composed of fructose and glucose through β-2, 1-glycosidic bond. It cannot be digested by mammal but is the most commonly modifier of microorganism (Moro and Boehm 2008).

7.2.4.4 Xylo-oligosaccharides (XOS)

Xylo-oligosaccharides are composed of 2–7 xylose molecules connected by β-2, 4-glycosidic bond and are the one of the functional oligosaccharides which have the most stable performance. Besides *Bifidobacterium*, the majority of intestinal bacteria in the human body have poor utilization of xylo-oligosaccharides.

7.2.4.5 The Physiological Functions of Prebiotics

1. Prebiotics promote the growth of *Bifidobacterium* and adjust the balance of intestinal flora.

Bifidobacterium is one of the main intestinal floras in human and colonizes in the gut from birth. The number of accounts for *Bifidobacterium* is about 80% in infant. This kind of bacteria would accompany with one person on lifetime, and the quantity in the intestine is closely related to the health of human (Bäckhed et al. 2005). It is accepted that *Bifidobacterium* is one of the most representative probiotics.

Bifidobacterium can largely breed by using nutrients in healthy human intestinal tract and form a physical barrier in intestinal mucosa surface to inhibit the pathogenic bacteria (e.g., enteroinvasive *Escherichia*, *Salmonella*, *Campylobacter*, *Listeria*). Clinical studies had shown that *Bifidobacterium* is nontoxic and is harmless to the human body. Besides its own metabolism and reproduction, *Bifidobacterium* also can eliminate free radicals, lipid peroxide, light free radicals, and some chemical substances by corruption bacteria such as amine.

Functional oligosaccharides, as a kind of ideal prebiotics in the human body, have excellent bifidogenic factors of physiological function (Roberfroid 2007b; Talwalkar and Kailasapathy 2004). Functional oligosaccharides ingested in the body can selectively stimulate the *Bifidobacterium* proliferation in human intestinal tract. *Bifidobacterium* can produce a large amount of short-chain fatty acids and other metabolites through fermenting functional oligosaccharides. When people get sick or suffer from malnutrition, it is commonly the intestinal tract's lack of carbon

source for beneficial bacterium. But the intestinal flora will change when the functional oligosaccharides which belonged to carbohydrates enter into the body. Because of the physiological structure of functional oligosaccharides, a variety of *Bifidobacterium* can exploit for different types of oligosaccharides in the intestinal tract. However, conditional pathogenic bacteria have relatively poor utilization ability of oligosaccharides (De Carvalho et al. 2006).

2. Elevate organic acids, reduce intestinal pH value, curb corruption, and prevent constipation.

The biggest difference between functional oligosaccharides and ordinary oligosaccharides is that the former cannot be absorbed by the human body. But functional oligosaccharides have special physiological function, because it can be used by intestinal bacteria to produce corresponding metabolic product. When the functional oligosaccharides enter into the intestinal tract, it can be utilized by beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* as carbon source. During the fermentation of *Bifidobacterium* and *Lactobacillus*, it will produce a large amount of short-chain fatty acid in the metabolic process, such as butyric acid, acetic acid, propionic acid, and lactic acid (Stewart et al. 2008).

These short-chain fatty acids can reduce the intestinal pH value and improve its osmotic pressure. Harmful foodborne pathogens will have a low fertility at low pH value. At the same time, poisonous intestinal putrefaction (e.g., formic acid, indoles, and benzene cresol) and harmful enzyme such as β-glucuronidase have low activity of generation and metabolism. The improvement of intestinal osmotic pressure can make the moisture absorbed by intestinal contents more fully and increase the fecal volume. In addition, it also can stimulate the intestinal fast screw to promote defecation and prevent constipation (Giacco et al. 2004).

3. Improve lipid metabolism, and promote the absorption of mineral elements.

The impact of functional oligosaccharides on body lipid metabolism research is still limited to fructo-oligosaccharides and isomaltooligosacharide. When the functional oligosaccharides is taken by the human body, the *Bifidobacterium* breed largely and produce the metabolites such as cholic acid hydrolase which can combine with free bile acid to inhibit the growth of pathogenic bacteria in the intestine. With the amounts of probiotic metabolism, bile acids can combine with cholesterol to generate cholesterol which can be out of the body with the intestinal contents when the pH is at 6. Besides, functional oligosaccharides have the function of adjusting blood fat and cholesterol (Li et al. 2004).

So much of functional oligosaccharides which can play a role on physiological depends on fermentation products from functional oligosaccharides and intestinal flora. Organic acids are produced when probiotic-fermented functional oligosaccharides and the intestinal pH are declined. The pathogens will also be declined in intestinal tract with secreting organic acids and other substances. Some compounds composed of calcium, phosphate, and magnesium are dissolved and absorbed on acidic intestinal environment. So, functional oligosaccharides can promote calcium, magnesium, zinc, iron, and other mineral element absorption.

4. Other physiological functions.

Functional oligosaccharides can promote intestinal *Bifidobacterium* proliferation. The *Bifidobacterium* can produce some kinds of B vitamins such as vitamins B1, B2, B6, and B12, folic acid, and nicotinic acid. In addition, the low pH value in intestines also can inhibit the growth of some vitamin decomposition microbes to enhance the vitamin content in the body.

Glucose transaminase, produced by *Streptococcus mutans* in the human mouth, cannot break down the oligosaccharide to glucose and galactose and fructose. It is the main reason that functional oligosaccharides can prevent dental caries.

7.2.4.6 The Mechanism of Oligosaccharides Regulating Intestinal Microbiota to Inhibit the Growth of Pathogenic Bacteria

Beneficial bacteria can get adequate nutrition and have a vigorous growth on proliferation under healthy human intestinal tract. In this case it also can restrain the growth of pathogenic bacteria. When the human intestinal flora disorder occurs, the harmful flora will proliferate largely and consume nutrients. At the same time, it will bring malnutrition to beneficial bacteria proliferation to make the intestinal environment increasingly harsh and break the balance of intestinal flora.

Under the condition of imbalance for intestinal flora after intake of oligosaccharides, intestinal *Bifidobacterium* and *Lactobacillus* bacteria lack of nutrition can selectively use oligosaccharides for metabolism and growth. Probiotics propagate heavily in the short term and inhibit the pathogenic bacteria to reply the balance of intestinal flora through intake of oligosaccharides continuously (Sertac Arslanoglu and Boehm 2007).

The possible mechanism of functional oligosaccharides on regulating intestinal flora is as follows:

1. The intestinal probiotics which ferment functional oligosaccharides will produce a large amount of short-chain fatty acids absorbed by the human body such as acetic acid and lactic acid. The breeding of harmful bacteria will be limited under acid condition. The short-chain fatty acid also can be absorbed by the intestine and involved in other human metabolisms as an energy source (Foreman-van Drongelen 1995).
2. It is reported that the exogenous lectins on bacteria surface could be associated with the specific sugar molecules of host epithelial cells. Molecular sugar, lipopolysaccharide, and glycoproteins could be used as a combination of pathogen receptors in the epithelial cell membrane. Because of the mannose and galactose contained into oligosaccharides, a part of pathogenic bacteria, except probiotics, would mistake the oligosaccharides for receptors under intake of oligosaccharides. The pathogenic bacteria could combine with oligosaccharides instead of the intestinal cell receptor analogues. In this case, the intestinal pathogenic bacteria would have affinity with oligosaccharides. The intestinal pathogenic bacteria combined with oligosaccharides would stay in the small lumen and not

adhesive on the intestinal cell walls. Lastly, the intestinal pathogenic bacteria would be drained out of the body with excrement. This is anti-infection protection mechanism of functional oligosaccharides (Goossens et al. 2005).

The role of probiotics and prebiotics is not mutually independent. Because of the cooperative effect of live probiotics and specific selective collaboration in prebiotics, the effect of combining probiotics with prebiotics at the same time is better than the single use of probiotics or prebiotics to intestinal health. Prebiotics, as an intestinal probiotic food, cannot be used by the harmful bacteria but can promote the growth and reproduction of beneficial bacteria. This character of prebiotics can promote the probiotics to become the competitive dominant bacteria to inhibit the growth of pathogenic bacteria. The cooperative work of probiotics and prebiotics makes the intestinal microecological balance to prevent and improve constipation and diarrhea. It is beneficial for the host to enhance immunity and keep the body healthy.

Inflammatory bowel disease (IBD) is a kind of the intestinal inflammatory disease including Crohn's disease (CD) and ulcerative colitis (UC) which is characterized by chronic inflammation. More and more research showed that the intestinal microbial flora imbalance plays a very crucial part on mechanism of IBD. It is reported that there was a significant difference on bacteria type and quantity in feces and intestinal mucosa between IBD patients and healthy controls (Lomasney and Hyland 2013; Alokail et al. 2013). Many IBD animal model studies confirmed that prebiotics certainly had the inhibitory effect of intestinal inflammation (Myung and Joo 2012). Recent research suggested that gut microbes could be adjusted to increase the tight junction protein expression to keep the integrity of the epithelial barrier when the host takes the dietary supplement prebiotics (Karahan et al. 2012).

Colorectal cancer (CRC) is a common gastrointestinal tumor. In recent years, more and more studies showed that probiotics and prebiotics would affect the occurrence and development of CRC. Probiotics and prebiotics will play a part by means of controlling and treating colorectal cancer. The prevention mechanism of prebiotics mainly includes enhancing immune response and inducing short-chain fatty acids.

7.3 The Application of Probiotics for Animal Health

7.3.1 Probiotics in Treatment of Animal Diseases

The disease of the animal husbandry and aquaculture will lead to the death of livestock or poultry. It will bring serious economic loss to farmers. The common type of animal diseases mainly can be divided into the following aspects: common disease, infectious diseases, and parasitic disease. Medicine, surgery, and obstetrics diseases are also included as common animal diseases. It has a higher incidence and diversified clinical symptoms. The epidemic and infectious diseases have certain

preclinical and clinical symptoms caused by pathogenic microorganisms. Viruses, bacteria, and fungi are likely to be the pathogens of infectious diseases. The clinical symptoms and pathological changes of animal infectious diseases also have a certain particularity and are difficult to prevent. The spread of disease caused by parasites such as arthropods, protozoa, and worms attacking against animal host is generally edible contain eggs or larvae of soil, water and feed. In addition to the harmful factors which exist in the growth or activity environment, a series of man-made factors can also cause disease in animals including unreasonable use of drugs in the process of breeding, farming equipment, and construction of imperfect and adverse farm management.

The reports of using *Lactobacillus* preparation to add into the livestock feed increase by years. Lactic acid bacteria have many effects on the host's health including adjusting the balance of animal intestinal microflora, enhancing the body's immunity and resistance, promoting the growth and development of the gut, inhibiting the growth of pathogenic microorganisms, keeping animal intestinal flora balance, and adjusting gastrointestinal digestion and absorption function. The lactic acid bacteria can effectively colonize, grow, and multiply in host intestinal tract which has formulation added into the animal feed after intake into the host. It also can form ecological competition sites to inhibit the growth of pathogenic bacteria and reduce the *Enterobacteriaceae* bacteria in each part of the gastrointestinal tract engraftment level. The lactic acid bacteria will play a great role in maintaining the body health through keeping the balance of animal intestinal microecological bacteria.

Probiotics are widely used in the process of pig breeding. Probiotics play a different role in the different growth stages of pigs. To pregnancy sow, the use of probiotics is mainly to improve the fodder digestibility or reduce constipation and stress. The feed added with lactic acid bacteria preparations can improve the quality and production of milk to lactation sows. It also can enhance the survival rate of piglets. For piglets, *Lactobacillus* used into fodder can improve the body weight and reduce diarrhea to increase the host immunity. In terms of improving feed utilization and meat quality or reducing diarrhea, probiotic compound preparation still shows some good advantage during the fattening stage. Weaning is a very complicated period; the food for piglets should transform from breast milk into plant feed. At the same time, the environment of piglets also is changed from delivery room to conservation of the group. In addition to the feed and hog house, the unsoundness of the piglet digestive system development may cause intestinal disease to piglets and economic losses to farmer. Zeyner (Zeyner and Boldt 2006) studies the effects of lactic acid bacteria microecologics to piglets. They found the *Enterococcus faecalis* feed for piglets could improve growth performance and reduce the incidence of diarrhea. Some scholar studies the *Bacillus subtilis* microecological function of piglets. The feed added with *Bacillus subtilis* promotes the body's growth performance and prevents diarrhea of piglet. At the same time, daily feed rates also were red intake and gains were increased significantly and diarrhea rate and feed conversions were reduced. Some scientists feed suckling pig with *Bacillus* CNCMI-1061 condensation. It was found that *Bacillus* CNCMI-1061 condensation can be able to colonize in intestinal bacillus and inhibit the growth of *E.coli* and other pathogenic

strains significantly. The results showed that the fodder added with *Bacillus* could observably promote the production performance and the intestinal flora balance stability. In addition, its role in improving host immunity or preventing and treating diarrhea was equally significant.

The probiotics added to fodder in young ruminant animals are mainly to promote the improvement of the rumen microbial flora, reduce ablactation stress, and decrease the harm of harmful bacteria. In dairy production, the use of probiotics aims to improve the milk quality and yield and augment the feed conversion rate. Besides, the role on probiotics in the improvement of the body weight gain and feed conversion rate is equally significant in beef cattle production. It is reported that *Propionibacterium jensenii* could increase the calf body weight gain and promote the development of rumen in daily ration. It also confirmed that the fodder added with probiotics can regulate the intestinal pH value and reduce the harm of acidosis.

The lactic acid bacteria also have the effective antagonist ability against pathogenic bacteria in poultry. Mountzouris et al. (2009) studied the treatment of probiotics added into the feed and potable water in chickens with enteritis caused by *Salmonella* compared with antibiotics. The 5 days chicken infected by 6×10^5 CFU *Salmonella* each one to form enteritidis were feeded with antibiotics and probiotic feed, respectively. 60 mg/kg salinomycin sodium and 2×10^9 CFU/kg BP5S were added into antibiotics and probiotic group fodder. The BP5S probiotics compound preparation included *Lactobacillus reuteri* isolated from healthy adult chicken crop, *Enterococcus* isolated from jejunum excrement, and *Bifidobacteria* and *Lactobacillus salivarius* isolated from ileum and cecum content, respectively. The result of the experiment showed that the BP5S probiotic compound preparation significantly reduced the morbidity and quantity of *Salmonella* in chicken body infected with pathogenic bacteria. It was close to the therapeutic effect of antibiotics.

Molnar et al. (2011) found the broiler's diet added with *Bacillus subtilis* could significantly improve body weight gain and feed conversion rate. In addition, the immune response also was increased by probiotic intervention. It is reported that poultry fodder added with 1000 mg/kg *Lactobacillus* compound also could increase the body weight gain and feed conversion rate. At the same time, the deposition of abdominal fat was reduced on 28-day detection. Probiotics also can enhance the utilization rate of feed, egg production, and egg quality during the production of laying hens. Mikulski et al. (2012) studied the efficacy of *Lactobacillus acidilactici* to laying hens. They added 100 mg/kg *Lactobacillus acidilactici* to laying hens' fodder. The result showed that *Lactobacillus acidilactici* increased the egg weight, eggshell thickness, relative weight of eggshell, and feed conversion rate. Besides, the cholesterol content of egg yolk was decreased as well.

7.3.2 The Application of Probiotics in Feed

The shortage of conventional feed has become the main factor of restricting the development of animal husbandry in modern agriculture-based animal husbandry. Digging the potential of existing feed resources and development of agricultural

by-products as nonconventional feed for adjusting measures to local conditions such as rice straw and green feed has been the main way to solve the feed shortage problem. *Lactobacillus* which mainly produce lactic acid could effectively inhibit the growth of pathogenic bacteria during the process of plant fermentation and give good flavor and texture to materials. The material after fermentation will become soft and have the aromatic acid and good palatability to livestock. It also can stimulate the appetite of livestock, promote the secretion of digestive juice, and increase the frequency of bowel movements to livestock. At the same time, the fermented feed also can enhance the host digestion function to prevent constipation. The increase of moisture and protein and decrease of fiber material after improving the nutrition of plant material properties during fermentation will improve the animal digestion of feed utilization rate. Lactic acid bacteria fermentation feed contains rich vitamin and has little damage of nutrient. The fermentation process which is controlled by manual intervention is not affected by natural factors such as weather and reduces the rate of pollution corruption. The metabolites during the feed fermentation can prevent the material deterioration to extend the shelf life of animal fodder and meet the needs of the winter forage grass shortage.

7.4 Conclusion and Future Perspectives

Probiotics have been extensively studied in in vitro and in vivo models. The potential application of probiotics as a means of controlling and treating enteric infections is reported. Healthy benefits of *Lactobacillus* include the prevention of diarrhea, atopic eczema, antibiotic-associated diarrhea, and traveler's diarrhea, prevention of dental carries and colorectal cancers, and treatment of IBD. However, as with any potential intervention strategies, probiotics also have a safety concern. Therefore, it is essential that metabolic and poisonous effects of lactic acid bacteria on humans are assessed for patient safety. The measure is especially for critically ill or immunocompromised patients. Significant challenges still exist in the effective application of probiotics in pathogen control. Future studies are essential to define best doses or their reasonable combinations of various lactic acid bacteria species based on their molecular mode of antimicrobial action. There is also a need for improvement of production techniques to understand and develop better approaches to probiotic delivery and biological availability in the gut as humans move from theoretical advantages to clinical application. Now with the possibility to express different molecules in probiotic bacteria, such as enzymes, cytokines, receptor mimics, adhesion molecules, antibodies, and host-targeting molecules, future research can help optimize applications and develop biologically contained strains to support clinical trials. Thus, probiotic bacteria can be used to control and prevent pathogen colonization in the food animals to improve food safety and a realistic therapeutic option in humans to control enteric pathogens.

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Chapter 8
Applications of Lactic Acid Bacteria in Heavy Metal Pollution Environment

Wei Chen and Qixiao Zhai

Contents

Abstract Heavy metals are kinds of metallic elements whose density is beyond 5 g/cm^3. Some heavy metals are essential trace elements, for example, iron, copper, and zinc, whereas others have no beneficial physiological function and may be toxic even in a very little amount. It is well known that cadmium, lead, mercury, and arsenic are the most toxic heavy metals. It is unavoidable that human beings are exposed to heavy metal in daily life. The range of adverse health effects induced by heavy metal exposure in humans and animals is broad. The common therapy used to deal with heavy metal intoxication is to promote the excretion by chelating. However, chelators themselves may have a lot of different safety and efficacy issues. Therefore, developing safe and effective methods against heavy metal toxicity is a necessary research field. Dietary strategies have advantages since they are easy and affordable to be added into the daily diet as the nutritional ingredients, including essential metal, vitamin, dietary phytochemical supplementation, and probiotics, and overcome the side effects of the chelation therapy.

Keywords Lactic acid bacteria · Alleviation · Heavy metal · Toxicity

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W. Chen, A. Narbad, *Lactic Acid Bacteria in Foodborne Hazards Reduction*,
https://doi.org/10.1007/978-981-13-1559-6_8

8.1 Introduction

Heavy metals are kinds of metallic elements whose density is beyond 5 g/cm^3. They have a number of inorganic and organic forms and are nondegradable compounds found naturally in the earth. Some heavy metals are essential trace elements, for example, iron, copper, and zinc, whereas others have no beneficial physiological function; in addition, they may be toxic even in a very little amount. For instance, cadmium (Cd) and lead (Pb) are the typical representations (Zoghi et al. 2014). It is well known that Cd, Pb, mercury (Hg), and arsenic (As) are the most toxic heavy metals (Halttunen 2008). There is an exponential increase in the releasing of heavy metals into the atmosphere, water, and soil as a result of the recent enlargement of human industrial activities, including mining, smelting, and synthetic compound creation (McConnell and Edwards 2008). Thus, it is unavoidable that the human beings exposed to heavy metal. Regulatory guidelines restricting heavy metal presence and exposure have been announced by many countries as well as remediation and treatment options. To prevent overconsumption, there are ongoing programs aiming to screen soil and water sources, but limitations such as unavailable programs and technologies exist especially in developing countries, where the burden is greatest (Ahsan et al. 2000; Li et al. 2006a, b). With the cycling of heavy metals in the environment, people around the globe are exposed, and it is urgent to reduce the adverse consequences of accumulation of heavy metals with new approaches. To give an example, in the surrounding environment, Cd and Pb are pervasive toxic heavy metals, and now the developing countries have the most severe Cd and Pb pollutions. Besides, blood lead in children is now considered to be unsafe at any level by the Centers for Disease Control and Prevention in the United States, as the threshold of BBL has reduced from 60 μg/dL in the 1960s to 10 μg/dL in 1991 to cause toxicity in children (Hassanien and El Shahawy 2011). With the expansion of pollution, the three bathes of blood lead detection were conducted with Chinese children in 2004, 2005, and 2006, and each time there were over ten thousand children who attended; the results showed that 10.10%, 7.78%, and 7.30% of children were over the threshold established in 1991 (Zhang et al. 2009). Cd from the earth normally accumulates in plant like rice which subsequently be consumed by human beings. In 2006, in the Cd-polluted sections of Jiangxi province in China, the average Cd levels of rice were 0.59 mg/kg, which is a 2.5 times growth since 1987 and exceeded the Chinese standard around 2 times (0.20 mg/kg) (Zhang et al. 2014). In 2007, the similar conclusion was drawn by a study performed in a Vietnam village which was heavy polluted, the Cd content in local rice was up to 0.31 mg/kg, whereas the standard level is 0.20 mg/kg, which was reported by the Vietnamese Ministry of Health (Minh et al. 2012). As groundwater is the natural pathway of As contamination, it is a global problem especially severe in India, Bangladesh, the United States, and Finland (Rahman et al. 2005; Smith et al. 2000; Ayotte et al. 2003; Kurttio et al. 1999). The International Agency for Research on Cancer (IARC) assigned drinking-water As in the group I human carcinogen (Humans et al. 2004).

In drinking water, the World Health Organization (WHO) has stipulated a recommendation level of 10 g/L which is provisional for As (Organization 2004).

The range of adverse health effects induced by heavy metal exposure in humans and animals is broad. The heavy metals are absorbed in the intestinal tract and then accumulate in many organs. Thus, the intestinal tract is the first organ impressionable to heavy metals (Nordberg et al. 2014). For the intestines, oral ingestion of Cd, Pb, Cu, and Al could lead to inflammation, epithelial cell death, and tight junction (TJ) protein dysfunction which result in disrupting the intestinal barrier and increasing toxic metal absorption (Breton et al. 2013b; Zhai et al. 2016a; Liu and Chen 2004; de Chambrun et al. 2014). The microbiota in gut, as a "forgotten organ," has a lot of beneficial functions, including the fermentation of unused energy substance, regulation of the immune system, and prevention of the growth of harmful bacteria (O'Hara and Shanahan 2006; Tremaroli and Backhed 2012; Pluznick et al. 2013). Several studies have shown that heavy metals are harmful to microorganisms in intestine, which is disrupting metal metabolism and inducing oxidative stress (Araúz et al. 2008; Bhakta et al. 2012a; Mikolay et al. 2010). Cd exposure can induce a significant reduction in the populations of representative intestinal microbial species and a decrease in the abundance of total intestinal bacteria in mice (Fazeli et al. 2011; Liu et al. 2014). High levels of oral Pb and Cu exposure lead to gut microbiota dysbiosis in rats, pigs, and humans (Namkung et al. 2006; Sadykov et al. 2009; Bisanz et al. 2014). The gut microbiota likely makes a strong influence in regulation of the bioavailability and toxicity of the heavy metals. A recent study found that germ-free mice are more susceptible to Cd and Pb than normal mice (Breton et al. 2013a). Zhai et al. studied the effects of toxic metal exposure, including Cd, Pb, Cu, and Al, on the gut microbiota through a microbiome analysis in mice (Zhai et al. 2017b). The results showed that long-term exposure to toxic metal would change the intestinal microbiota of mice in a metal-specific and time-dependent manner. Cd toxicity includes dysfunctions in the lung, kidney, liver, bone, and reproductive system (Lauwerys et al. 1974; Hong et al. 2004; Koyu et al. 2006; Murata et al. 1970; Rehm and Waalkes 1988; Tellez-Plaza et al. 2008). The IARC classified Cd which is nonessential in the group I human carcinogen (Bickers and Mukhtar 1994). Neurologic and hematological dysfunctions can be induced by Pb exposure, besides damage in the kidney and liver, and disorders of the reproductive system in the human body can be boosted (Lidsky and Schneider 2003; Bergdahl et al. 1998; Sandhir and Gill 1995; Fowler et al. 1980; Ronis et al. 1996). Being exposed to As in a long term would usually result in skin lesion (pigmentation changes and then hyperkeratosis). When the exposure to As lasts for 10 years, cancer of the skin, lung, bladder, and kidney may develop (Gomez-Caminero et al. 2001). The interactions between heavy metals and essential metals, together with the oxidative stress induced by heavy metals, are considered to be the main reasons of heavy metal toxicity among all the reported routes (Ahamed and Siddiqui 2007; Vesey 2010; Farmand et al. 2005; Liu et al. 2009). These two mechanisms have overlaps and interrelations since some basic metals such as Zn and selenium (Se) can also damage the oxidative and antioxidative systems of the body with accurate intake amount (Oteiza et al. 1995; Brenneisen et al. 2005).

The common therapy used to deal with heavy metal intoxication is to promote the excretion by chelating. However, chelators themselves may have a lot of different safety and efficacy issues. Therefore, developing safe and effective methods against heavy metal toxicity is a necessary research field. It is reported that dietary supplements have a vital effect in alleviating or preventing heavy metal toxicity. Dietary strategies have advantages since these are easy and affordable to be added into the daily diet as the nutritional ingredients and overcome the side effects of the chelation therapy. Therefore, we can evaluate the potential dietary strategies, including essential metal, vitamin, edible plant, and dietary phytochemical supplementation and probiotics, to alleviate heavy metal toxicity (Fig. 8.1).

When we define probiotics, normally we quote the one that was given by the WHO, "live micro-organisms which confer a health benefit on the host when administered in adequate amounts," (Hotel 2001). *Bifidobacterium*, *Bacillus*, *Lactobacillus*, and *Saccharomyces boulardii* as probiotics are widely applied in commercial products (Foligne et al. 2013). With its safety and effectiveness, probiotics are now a profitable industry. Numerous studies have indicated probiotics are characterized with promising benefits in improving diarrhea allergy, lactose intolerance, and high blood cholesterol level which are associated with antibiotic and they can protect against gut pathogens by developing the immune system (Jankovic et al. 2010; Rijkers et al. 2010). Besides, many toxic compounds can be bound by probiotic strains, such as aflatoxins (Peltonen et al. 2001; Haskard et al. 2001; El-Nezami et al. 2006) and mutagens broadcasted by food (Turbic et al. 2002; Orrhage et al. 2002) in vitro and in vivo. With evidences from the studies of *Lactobacillus rham-*

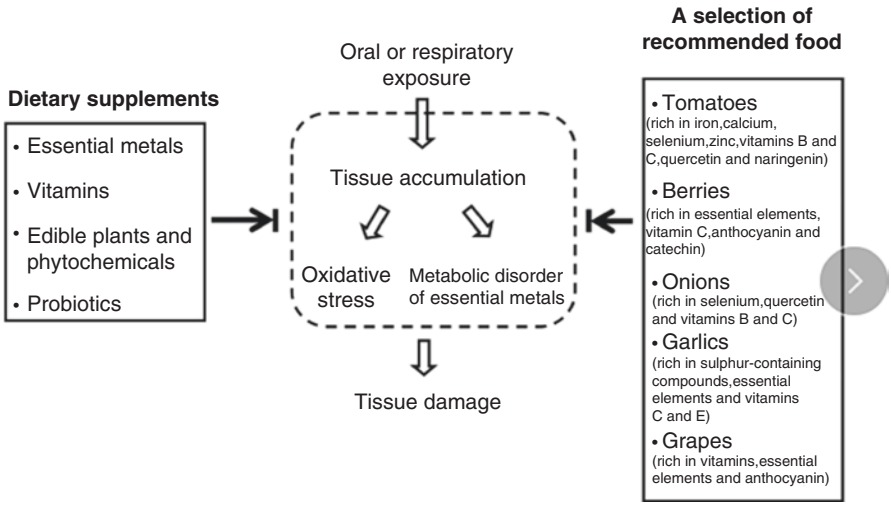

Fig. 8.1 The alleviation of heavy metal toxicity by dietary supplements and recommended strategy

nosus, *L. plantarum*, and *Bifidobacterium longum* strains, it can be concluded that heavy metals, such as Pb, Cu, and Cd, could be bound by lactic acid bacteria (LAB) in vitro (Halttunen et al. 2007a, b; Ibrahim et al. 2006; Zhai et al. 2015c; Tian et al. 2012, 2015). Bisanz et al. have showed the promising value of the long-term intake of *L. rhamnosus* GR-1, as an intervention, in relieving mercury (Hg) and arsenic (As) exposure in vulnerable populations, particularly in pregnant women (Bisanz et al. 2014). Kinoshita et al. believed by the oral intake of LAB, it is not only capable to reduce the absorption of heavy metals into the body but also to clear heavy metals out of the body in high performance during defecation (Kinoshita et al. 2013). Moreover, in human body, LABs have antioxidative properties (Kullisaar et al. 2003; Ejtahed et al. 2012), which are valuable for the protection from heavy metal toxicity. It was found by studies that specific *L. plantarum* strains could reduce intestinal absorption of heavy metal, decrease heavy metal accumulation in the tissue, alleviate tissue oxidative stress, and ameliorate tissue damages without loss of essential metals (Zhai et al. 2014; Tian et al. 2015). With these functions, specific LABs are potential to be applied in against heavy metal toxicity.

8.2 Heavy Metal Removal by Lactic Acid Bacteria In Vitro

8.2.1 Heavy Metal Tolerance

Since heavy metal pollution spreads nonbiodegradable, hazardous, and toxic properties into the environment, it is not only a hazard to the human health but also a risk to the broader environment (Bhakta et al. 2012b). For example, heavy metal pollution also unbalances the environmental microbial community marvelously. When the soil microbial community are affected by heavy metal pollution, metabolic activity and diversity would be decreased (Giller et al. 1998), so it requires the applied probiotic strain against heavy metal pollution. Thus, it is reasonable that Bhakta et al. isolated lactic acid bacteria (LAB) that are resistant to Pb and Cd from heavy metal-contaminated habitats in the environment (Bhakta et al. 2012b). The minimum inhibitory concentration (MIC) approach was used to determine the heavy metal tolerance of LAB strains (Abou-Shanab et al. 2007). MIC was defined as the minimum concentration of heavy metal to absolutely inhibit the growth of the bacteria. Twenty-six of 255 isolated strains that are resistant to Pb and Cd were identified from 53 environmental samples. They varied from 60 to >1000 mg/L of all the 26 LAB strains in Cd MIC values, whereas MIC values against Pb were remarkably high (>2000 mg/L) for all the evaluated LAB strains. Zhai et al. investigated the MIC values for Cd of 11 LAB strains (Zhai et al. 2015c). MRS agar medium was prepared with sterilized Cd chloride solution with different final concentrations. Four parts were divided equally in every agar plate, and each sector was spotted delicately with precultured LAB strain. Each Cd concentration agar plate

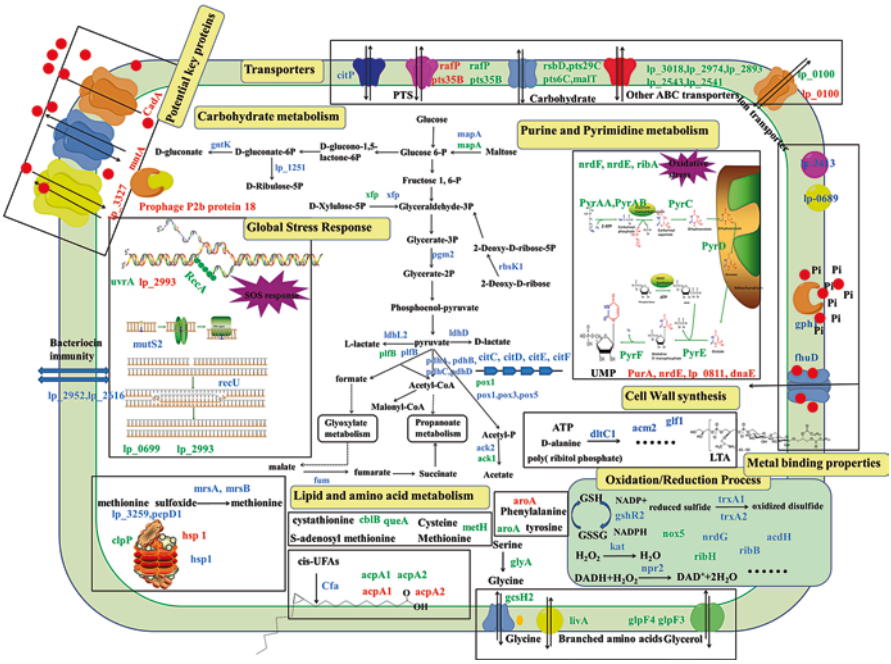

Fig. 8.2 The response mechanisms of CCFM8610 to Cd stress

was also inoculated in duplicate. After the incubation, the growth of bacteria was observed and recorded. The MIC was defined as the lowest concentration of Cd to absolutely inhibit the growth of the LAB. The result indicated that the Cd-resistant abilities of strains are quite different. It is defined that the strain is tolerant to Cd, whose MIC value is higher than 100 mg/L (Akinbowale et al. 2007; Matyar et al. 2008) or 112.4 mg/L (Abou-Shanab et al. 2007). Per this definition, there are five strains—two *L. plantarum* strains, two *L. rhamnosus* strains, and one *B. bifidum* strain—found to be Cd tolerant. Moreover, compared with the other tested strains, *L. plantarum* CCFM8610 had the highest MIC value. In order to find out the underlying Cd tolerance mechanism of the intraspecies differences of *L. plantarum* strains, Zhai et al. used the iTRAQ approach to examine the proteomic profiles of CCFM8610 (with strong Cd-resistant ability) and CCFM191 (sensitive to Cd) in non-stimulating condition and Cd exposure condition (Zhai et al. 2017a). The results indicated that *L. plantarum* CCFM8610 responded to Cd stress by a complex biological network, which might be associated with carbohydrate, purine, and pyrimidine metabolism, global stress responses, lipid and amino acid metabolism, metal binding properties, cell wall biosynthesis, and transporters of the bacterial cell (Fig. 8.2). The mechanism of this strain to resist Cd may include a specific mode of energy conservation survival, a mildly induced cellular defense and repair systems, a increased biosynthesis hydrophobic amino acids, an excellent tolerance against osmotic stress, a superior inherent Cd-binding ability and a effective cell

wall biosynthesis ability in response to Cd stress. Previous studies suggested that if MIC value of strain was over 500 mg/L, it can be regarded as Al-tolerant strain (Abo-Amer et al. 2012; Ozdemir and Baysal 2004). Based on this standard, Yu et al. investigated the MIC values for Al of 18 LAB strains. It turned out that all the tested strains hold different MIC values. Eleven of the measured strains were identified as tolerant to Al, and *L. plantarum* CCFM639 with the highest MIC value, higher than 2048 mg/L.

Altered proteins (fold change >1.5 or <−1.5 and $P < 0.05$) are presented above in three comparisons; they are CCFM8610(0)/CCFM191(0) (blue), CCFM8610(Cd)/CCFM8610(0) (red), and CCFM191(Cd)/CCFM191(0) (green). Red dot represents Cd ion.

The growth curves of the 16 LAB strains cultured in the MRS medium with various amounts of copper ions were investigated by Tian et al. (unpublished data) (Tian et al. 2015). The dose of copper ions is 0, 50, 150, and 500 mg/L, respectively. The incubation lasted for 25 h, and samples were taken every 2 h to measure the OD_{600} values to represent the cell density values. Three types of growth patterns can be recognized. The first one was that strains could not grow in MRS broth with 50, 150, or 500 mg/L copper ions generally for that they were very sensitive to copper. The second one was that no obvious growth inhibition was observed and the growth status of strains was similar no matter the concentrations of copper ions. The last one was that strains were affected differently by different concentrations of copper ion during their growth.

Some researchers found that many bacteria isolated from soil have a superior ability of tolerating and mobilizing heavy metals (Gadd 1990; Idris et al. 2004). Bhakta et al. isolated metal-removing LAB from the bacterial community that are resistant to metals and naturally existed in the environment. They isolated LAB resistant to Cd and Pb from the sediments of coastal aquaculture habitats by spread plate techniques with Cd or Pb at 50 mg/L and then used 16S rDNA sequencing to identify them, indicating they are the probiotic with the potential to remove metal (Bhakta et al. 2012a). Finally, they gave a conclusion that isolated metal-resistant *E. faecium* Pb12 strain, whose MIC values are 120 mg/L for Cd and 800 mg/L for Pb, might be used as a potential probiotic strain for clearing heavy metals in fish intestine and controlling the accumulations of heavy metals in fish.

8.2.2 Heavy Metal Binding

Air, water, food, and industrial materials and products are carriers of heavy metals that exposed the general public (Rozman and Klaassen 2007) and are hard to avoid. Fortunately, new solutions might exist with some studies that have tested that lactobacilli and other potentially probiotic bacteria can bind metals preventing their entry to the body and thus protecting the host (Monachese et al. 2012). Removal of toxic metals using probiotics has been recommended as an inexpensive, safe, and novel method besides the conventional methods (Zoghi et al. 2014).

Binding or removing abilities of LAB have been reported for Cd, Pb, and Cu in vitro (Zhai et al. 2015a, b, c; Tian et al. 2012, 2015). Zhai et al. demonstrated an ability of 33 LAB strains to bind Cd that is concentration-dependent when the initial Cd concentrations are 5, 50, and 100 mg/L, respectively (Zhai et al. 2015a, b, c). The maximum percentage removal was found in *L. plantarum* CCFM8610 (about 61%, 31%, and 24% for three different initial Cd concentrations). They also observed that Cd was deposited on the surface of the cells obviously after binding and no Cd was found in untreated cells by TEM and SEM micrographs (Figs. 8.3 and 8.4) (Zhai et al. 2016b). They also found an extra Cd peak in Cd-treated cells confirmed by EDX spectra, and the peak wasn't found in the control sample. The SEM micrographs showed that it caused anomalous aggregation of the *L. plantarum* CCFM8610 cells when exposed to Cd, but they found no morphological changes in the control biomass. The morphological alterations of the strain and the anomalous aggregation after Cd binding may be due to the change in surface charge and the proteins' degeneration, so that the Cd-binding ability of the cells was enhanced. As shown in Fig. 8.5, the ability of *L. plantarum* CCFM8610 to remove Cd by a method to plot the amount of Cd bound by the bacteria (qe) versus the equilibrium Cd concentration of the metal (Ce) was evaluated. The Cd-binding ability gained along with the raise of Cd concentration in the solution that finally reached to the equilib-

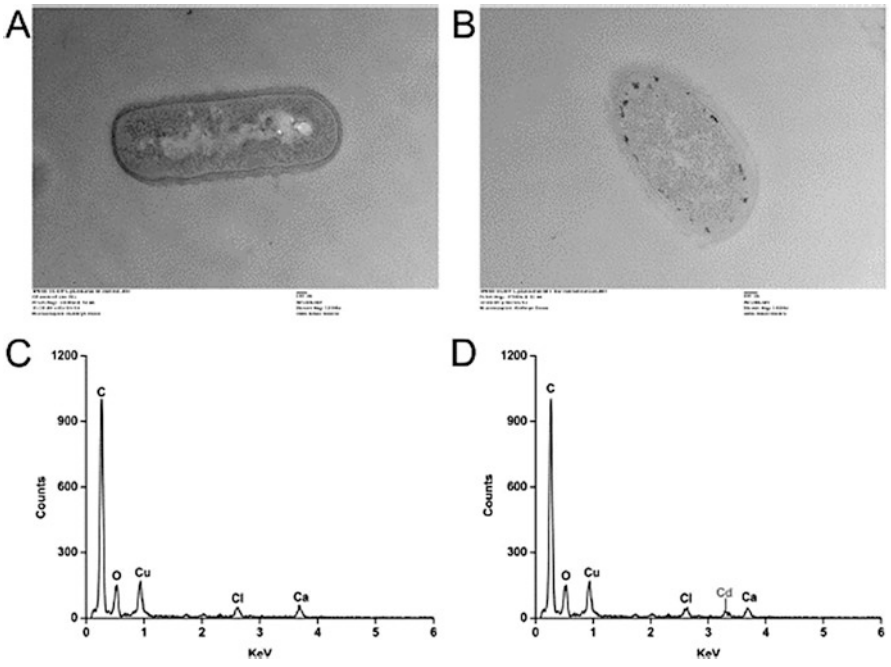

Fig. 8.3 The images of *L. plantarum* CCFM8610 before and after Cd binding using transmission electron microscopic. (**a**) Untreated cells. (**b**) Cells after Cd binding. (**c**) EDX spectra of untreated cells. (**d**) EDX spectra of cells after Cd binding. Scale bar 1/4 100 nm

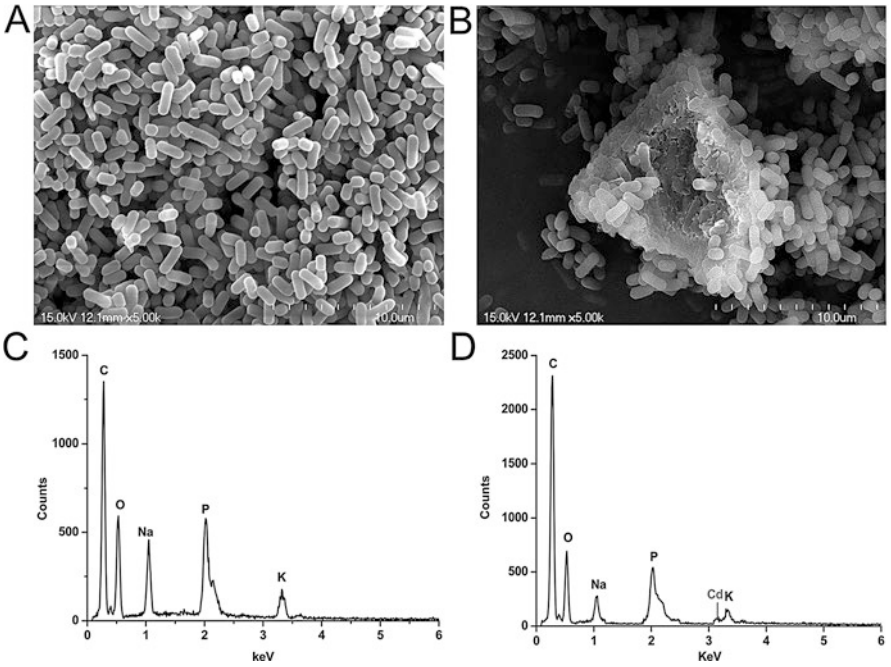

Fig. 8.4 The images of *L. plantarum* CCFM8610 before and after Cd binding using scanning electron microscopic. (**a**) Untreated cells. (**b**) Cells after Cd binding. (**c**) EDX spectra of untreated cells. (**d**) EDX spectra of cells after Cd binding. Scale bar 1/4 10.0 mm

rium value. They used different isotherm models to make a further analysis (Table 8.1). The Langmuir-Freundlich is the fittest model to the Cd biosorption by the strain as the highest R^2 value ($R^2 = 0.9928$). This is consistent with the heavy metal binding characteristic of other strains, which indicated the contribution of physical and chemical binding mechanisms (Chakravarty and Banerjee 2012). The result of kinetic analysis indicated a rapid binding process which best fits the pseudo-second-order rate model, indicating the involvement of chemical absorption during the binding process (Srivastava et al. 2006; Bueno et al. 2008).

Tian et al. compared the Pb-binding abilities of nine lactobacilli and found that both living and dead *L. plantarum* CCFM8661 had a very good capacity to bind lead with the biosorption of 49.551 mg/g and 53 mg/g dry biomass, respectively (Tian et al. 2012). Intriguingly, the investigation of Cu-binding capability for 16 strains showed that, in line with previous studies (Tian et al. 2012; Zhai et al. 2013; Aksu and Dönmez 2001), all living bacteria were higher than the dead ones (Tian et al. 2015). It's considered that physical and chemical adsorption, as well as ion exchange of cell surface, relates to the Cu biosorption of lactobacilli (Chakravarty and Banerjee 2012; Montazer-Rahmati et al. 2011; Halttunen et al. 2007a) and boiling-induced irreversible cell structure damage; reducing binding sites for metal irons may result in the differences between the living and the dead. Kinoshita et al.

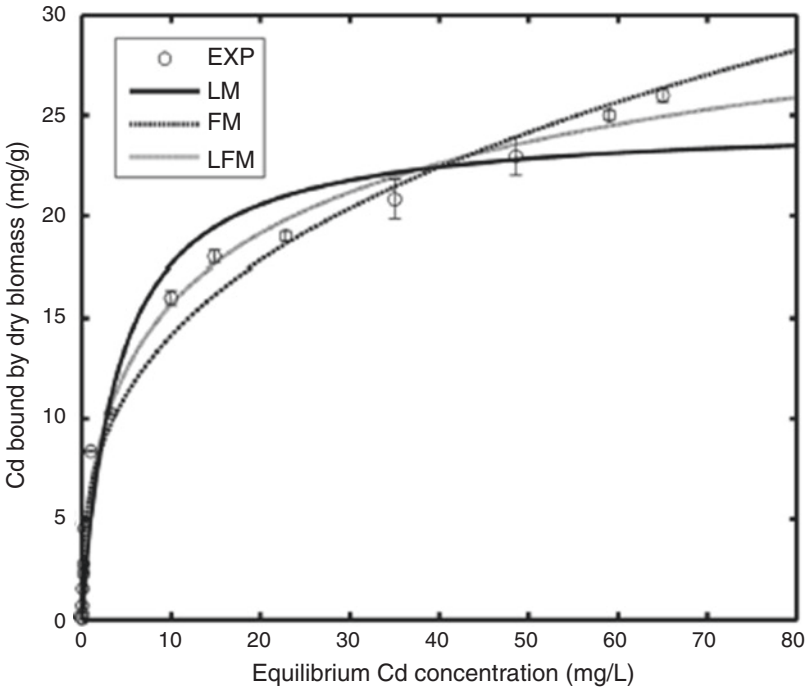

Fig. 8.5 Isotherm of Cd binding adsorbed by *L. plantarum* CCFM8610. *EXP* experimental data obtained in the present study, *LM* Langmuir model, *FM* Freundlich model, *LFM* Langmuir-Freundlich model. The form of Cd in aqueous solution is cadmium chloride. Values are presented as mean ±SEM

Table 8.1 Simulations of biosorption constants of three isotherm models[a]

Isotherm models	Constants	
(1) Langmuir	Q_{max}	24.69 (mg g^{-1} dry biomass)
$q_e = \frac{Q_{max} b_L C_e}{1 + b_L C_e}$	b_L	0.2494
	R^2 (nonlinear)	0.9679
(2) Freundlich	K_F	6.575
$q_e = K_F C_e^{1/n_F}$	n_F	3.006
	R^2 (nonlinear)	0.9834
(3) Langmuir-Freundlich	K_{LF}	7.941
$\text{dual} q_e = \frac{K_{LF} C_e^{1/n}}{1 + a_{LF} C_e^{1/n}}$	a_{LF}	0.2038
	n	1.926
	R^2 (nonlinear)	0.9928

[a]qe (mg/g) represents the equilibrium content of cell binding Cd. Ce (mg/L) represents the equilibrium Cd concentration

investigated the biosorption of Pb, As, and Hg for the selected 11 LAB strains, and the results showed that all strains had a satisfied Hg biosorption capability over 85% and the highest biosorption (99.1%) was observed in *L. sakei* MYU 10 (Kinoshita et al. 2013). However, biosorption capabilities for Pb and As were all relatively weak; the highest biosorption rates for these two metal irons were 29.2% and 14.8% (observed in *L. casei* MYU 49 and *L. sakei* MYU 10, respectively). Yu et al. compared the Al-binding capacity among the 30 strains and found that the removal rates were all over 20% in 17 strains with the initial Al concentration of 5 mg/L (Yu et al. 2016a). And *L. plantarum* CCFM639 exhibited the highest Al removal capability. Furthermore, they also performed the binding test for the 17 strains mentioned above to investigate if higher removal ability is related to a higher binding capability. Thus, the experiments were conducted under the initial Al level of 50 mg/L, and the results definitely substantiate the hypothesis. Similarly, *L. plantarum* CCFM639 possessed the best binding capacity with an Al binding rate of 64.54 and 26.83 % at 5 and 50 mg/L initial Al levels, respectively.

The involved mechanisms of heavy metal binding by different bacteria may be complex and still not fully understood (Tian et al. 2012). It was reported that the possible mechanisms involved in metal biosorption include complex formation, ion exchange, complexation, and microprecipitation (Halttunen 2008; Gadd and White 1989). The binding of LABs to cationic heavy metals depends partly on the strain and pHs. The removal rates of Cd and Pb are typically very low at pHs below 2–3, whereas a sharp increase occurs at pHs above 3 and maximum removal is often observed at pHs 4–6. The competition for negative charge binding sites between cationic metals and protons is one of explanation for the effect of pHs on metal iron removal ability of LAB (Halttunen 2008). Harvey RW et al. found that Cd- and Pb-binding abilities enhanced with increasing cell concentration, while the further increase induced a decreasing Cd or Pb binding for the reason that organics produced by cells accumulated significantly in extracellular matrixc, competed for binding sites, and resulted in cell aggregation (Harvey and Leckie 1985). Anionic groups on the cell surface also play important roles in metal binding. As Gram-positive bacteria, the surface of lactobacilli cells is covered by a thick peptidoglycan sacculus decorated with proteins, teichoic acids, and other extracellular coatings such as capsular polysaccharides, which is quite different with Gram-negative bacteria (Huang et al. 1990). There are different kinds of charged groups on the cell surface of Gram-positive bacteria including hydroxyl and phosphate groups, and the abilities of lead binding decrease when carboxyl and phosphoryl groups, both the negative charges, are neutralized, suggesting that these two kinds of surface groups play specific roles in the binding of heavy metals (Hao et al. 1999). This can well explain the significant difference of Pb-binding efficiency between *L. plantarum* CCFM8661 and *E. coli* ZH2133 in the previous study, which reported a significant decrease in the Cd- and Pb-binding abilities of *L. fermentum* ME3 and *B. longum* 46 when the negative charges of –COOH and $–PO_3H_2$ were neutralized (Tian et al. 2012). Both of these two groups influence the binding effects of heavy metals, and they may act as ion exchange sites. In addition, the removal of Cd and Pb by *L. rhamnosus* GG was influenced weakly by temperature (Halttunen 2008). However,

heat treatment increases Cd removal in most cases, and this kind of heat-caused enhancement is partly due to the increase of binding site availability on the cell surface (Goksungur et al. 2005). What's more, it is known that heat or ethanol treatments can fix soluble cell wall proteins to the cell surface; otherwise the proteins can be solubilized and compete with surface binding sites (Huang et al. 1990). Two phase kinetics are observed in Cd and Pb removal curve: initially rapid binding rate and then a lasting slow, nearly constant removal for hours. Previous study suggested that, in the first phase, the outermost structures of the lipopolysaccharide (LPS) layer would bind Pb and then further diffusion into the inner LPS layer occurred slowly in the second phase (Harvey and Leckie 1985). Besides, energy-dependent accumulation of Cd has been reported in *L. plantarum* (Hao et al. 1999). What's more, rapid bound on the bacterial surface is hindered under the presence of other cationic metals, which may due to electrostatic interactions occurred between these cationic metals and cell wall components. Finally, a recently research showed the reversibility binding and full desorption of bounded Cd and Pb by HNO_3 or EDTA. And removal of two metals was significantly decreased when regenerated biomass of EDTA was used (Halttunen 2008).

Kinoshita et al. investigated the Hg-binding proteins of the cell surface by Hg column assay (Kinoshita et al. 2013). A 14 kDa protein was identified, and it contains a CXXC motif (X is any amino acid) by the N-terminal amino acid sequencing. CXXC motif is widely regarded as a heavy metal-binding motif with binding capabilities for Cd, Co, Cu, and Zn ions, even though the definite function is unknown. Other proteins such as CopA, CopZ, and McsA in *S. aureus* also have CXXC motifs and been reported having the capability to bind various types of heavy metals (Kinoshita et al. 2013; Sitthisak et al. 2007, 2012). Roesijadi et al. found another two Hg-binding proteins (HgBP) with relatively low molecular weights as well as abundant proportions of halfcystine (26%) and glycine (16%) (Roesijadi 1986). Metallothionein (MT), a well-known protein with the ability to bind heavy metals (Nordberg et al. 1972; Weser et al. 1973), was reported that it has advantages in maintaining intracellular ion homeostasis and contributing to the detoxification action for heavy metal ions (Kinoshita et al. 2013). Previous researches showed that the affinity of MT to the heavy metals ranks in a descending order as follows: Hg ≥ Ag > Cu > Cd > Pb > Zn. And in line with this trend, Kinoshita et al. found that biosorption of LABs for diverse heavy metals ranks as Hg > Cd > Pb and As (Kinoshita et al. 2013). Some researchers also reported an elevating tissue MT levels in humans and animals with accumulation of Cd (Järup and Åkesson 2009; Zhang et al. 2012; Banni et al. 2010). In addition, the role of MT in protection of humans and animals against Cd toxicity is of crucial importance (Klaassen et al. 2009). MT not only binds and detoxifies Cd in tissues but also has effective free radical scavenging ability which can protect against Cd-induced oxidative stress (Cherian and Kang 2006; Min et al. 2005; Kara et al. 2005). Zhai et al. also found the increasing MT protein level in the liver of Cd-treated mice. More importantly, compared with the Cd-only group, the co-treatment group (both Cd and CCFM8610 were administrated) has a decreasing MT protein level in the liver, which may result from a reduced Cd uptake caused by CCFM8610.

The lactic acid bacterium 70810, isolated from Chinese paocai (a cabbage-fermented food), could produce a novel EPS and was identified as *L. plantarum* (HQ259238) (Feng et al. 2012). Conditions for the maximum Pb adsorption by 70810 EPS were optimized as follows: pH 5, 30 °C, and 6 h. In a test assay with a volume of 5 mL, the metal uptake elevated from 25.44 to 160.62 mg/g when 70810 EPS increased from 0.5 to 1 mg. However, when the dosage continues to increase, the metal uptake decreased. Mechanisms underlying this Pb-binding ability of EPS were explained by SEM and FI-IR analysis. The change of surface morphology, as well as various EPS functional groups including –OH, COO–, C=O, and –NH, involved in this process. Magdalena et al. demonstrated that functional groups of *Lb. rhamnosus* E/N EPS are associated to its Cd^{2+} and Al^{3+} removing ability in water solutions (Polak-Berecka et al. 2014). Pérez JAM et al. believed that biosorption process here is metabolic independent (Pérez et al. 2008). Acidic functional groups of EPS with negative charges mutually interact with metal cations, involving in it. Carboxyl, acetate, hydroxyl, amine, phosphate, and even rarer sulfate groups in EPS are all ionizable and can be potential binding sites (Liu and Fang 2002). Thus, EPS isolated from *Lb. rhamnosus* E/N can be a promising tool for the removal and detoxification of heavy metals in the gastrointestinal tract.

8.2.3 Heavy Metal Removal from Fruit and Vegetable Juices

Heavy metal pollution in agricultural soils causes several food safety concerns, potential health risks for both humans and animals, as well as the enormous damage on soil ecosystems and has received widespread attention (McLaughlin et al. 1999; Cui et al. 2004). The heavy metals in the soil are partly derived from parent materials and partly from anthropogenic activities (Cui et al. 2004). They have been also detected in various fruits and vegetables, including apples, tomatoes, pears, celeries, carrots, cucumbers, and so on, because of their high transformation rate from the soil to plants (Cui et al. 2004; Yang et al. 2009; Krejpcio et al. 2005). Although the level in fruits and vegetables are relative low, a cumulative effect caused by their high consumptions can't be ignored (Tahvonen 1998). Many studies have been focusing on the health risk of heavy metal pollution in soils. Some researchers showed that in the contamination areas of Japan (Ryan et al. 1982) and China (Shiwen et al. 1990; Nordberg et al. 1997; Watanabe et al. 1998, 2000; Jin et al. 2002), lifetime exposure to Cd in a low level could cause renal dysfunction for the local residents. Recently, lactic acid bacteria (LAB), which includes *Lactobacillus rhamnosus*, *L. plantarum*, and *L. reuteri*, have been reported to have binding and removing potential to the heavy metals like Cd and Pb in vitro (Bhakta et al. 2012a; Zhai et al. 2015c; Tian et al. 2012; Halttunen et al. 2007b). Oral administrations of *L. rhamnosus* to the pregnant women, who are suspected to have high toxic metal exposures, can prevent the increases of Hg and As in the blood (Bisanz et al. 2014). Zhai et al. also confirmed the Cd sequestration function of *L. plantarum* CCFM8610 in the intestines, which can decrease the Cd absorption in mice with

chronic dietary intake of Cd, even though the underlying mechanism has not been elucidated currently (Zhai et al. 2014).

L. plantarum strains, which have been used in various aspects of the food industry, such as lactic acid fermentation, are commonly considered as safe and efficient to improve nutritional and sensory values and extend the shelf life of fruit and vegetables (Foligne et al. 2013; Filannino et al. 2013, 2015). Zhai et al. investigated *L. plantarum* CCFM8610, a probiotic with relatively good Cd-binding properties, and assessed its potential application in fruit and veggie juice to protect human against Cd exposure (Zhai et al. 2016b). In their study, viable bacterial cells of *L. plantarum* CCFM8610 were added to the fruit and veggie juice including apples, cucumbers, and tomatoes harvested from the contaminated area in China with the final concentration of 10^7 cfu/mL, and the colonies were counted after incubation (Filannino et al. 2013; Pereira et al. 2011). After that, the supernatant was collected for Cd content measurement by flame atomic absorption spectrophotometry, and the results revealed that Cd removal rate in different juices varied from 56.18% to 81.79% after 36-h treatment of *L. plantarum* CCFM8610. Further research showed that 40.54% ± 0.80% of Cd accumulates on the outside of cell wall and 54.30% ± 2.93% of Cd is located between the cell cytoplasmic membrane and the cell wall. The Cd entering the protoplast is only approximately 7%. Besides, the research also showed that when some functional groups located in the cell surface of *L. plantarum* CCFM8610 were modified, its Cd-binding ability would be hampered. For example, the methylation of amino groups in the cell surface was related to the decreasing Cd-binding ability from 48.16% to 5.95%, and this kind of decrease was also observed when carboxyl groups were neutralized. Cd biosorption characteristic study of *L. plantarum* CCFM8610 showed its process, which is fast, efficient, and pH-dependent, closely obeys the Langmuir-Freundlich isotherm, the dual-site Langmuir isotherm, and pseudo-second-order kinetics model. These results indicate that these food-grade bacterial strains can be potentially used in fruit and vegetable juices to remove heavy metals.

Zhai et al. also investigated the consequences of soymilk fermentation with the addition of *L. plantarum* CCFM8610 and *L. bulgaricus* CCFM8004 on the protection against chronic Cd exposure of mice and illustrated the cooperative effects between these two lactic acid bacteria and soymilk (Zhai et al. 2015b). The study also shows that the protection effects of fermented soymilk by *L. plantarum* CCFM8610 against chronic Cd-exposed mice are more significant than non-fermented soymilk. This fermented production has an ability to increase the excretion of Cd in the feces, reduce the concentration of Cd and oxidative stress in mice tissue, reverse the damage biomarkers in liver and kidneys, and alleviate histopathological changes in mice tissue.

We also draw the conclusion that chronic Cd-exposed mice fed by soymilk fermented with specific mixed lactic acid bacteria show a better protection effect than with lactic acid bacteria alone or unfermented soymilk. The better protection effect provided by fermented soymilk may be due to the conjunct action of soymilk and the specific lactic acid bacteria strain in the fermentation process (Fig. 8.6).

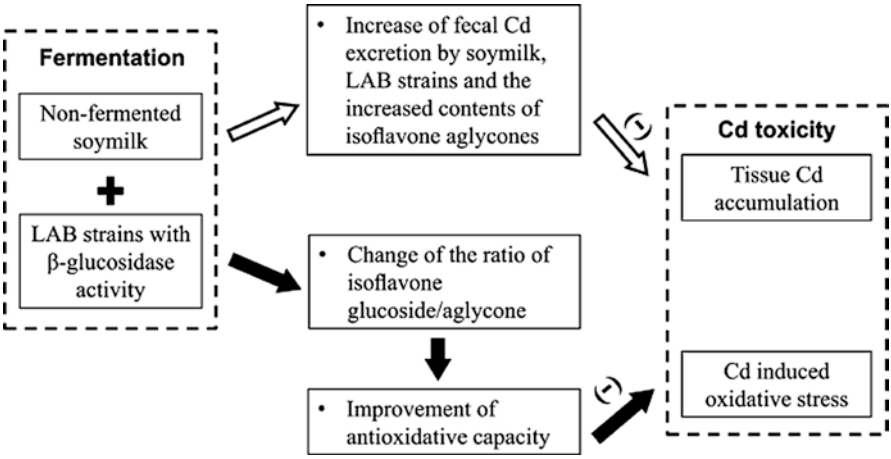

Fig. 8.6 The proposed conjunct influence of soymilk and the LAB strains in the fermentation procession under the chronic Cd exposure

8.3 Heavy Metal Toxicity Alleviation by Lactic Acid Bacteria In Vivo

8.3.1 Decrease of Heavy Metal Accumulation

Heavy metal accumulation is becoming a serious worldwide health problem with the development of industry; people are exposed to heavy metals by various invisible methods, such as the principal use of Cd batteries, pigments, fume, and commercial fertilizers (Budak et al. 2014; Klaassen et al. 2009). Many researchers reported that heavy metal is a persistent environmental pollutant which slowly accumulates leading to bioaccumulations in food chains and cause a variety of adverse effects to human beings (Naik and Dubey 2013). For example, heavy metals have been acknowledged to accumulate in the body of various aquatic animals, especially fish, which result into the heavy metal accumulation in human body (Cheng and Gobas 2007). Cd accumulation has also been detected in diverse fruit and vegetable, such as apples, pears, peaches, tomatoes, and cucumbers, because of the high rate transfer of heavy metals (Coco et al. 2006; Muñoz et al. 2005; Krejpcio et al. 2005). Despite of low Cd accumulation in fruit and vegetables now, Cd exposures can still be the fact by a huge intake of fruit and vegetables or fruit juices (Tahvonen 1998). Therefore, decreasing the amount of heavy metals from the environment and food is an indirect way of avoiding heavy metal accumulation in animals and humans, and the direct way is reducing the accumulation in vivo (Bhakta et al. 2012b). Among all the in vivo methods, chelation therapy which could promote the excretion can alleviate toxic metal intoxication effectively and the most defectively. Metal chelators that are in use, such as dimercaptosuccinic acid (DMSA) and ethylenediaminetetraacetic acid (EDTA), decrease heavy metal accumulation

acutely, but they are not suitable for long-term use in chronic cases (Bisanz et al. 2014). Antioxidants can reduce oxidant stress induced by alleviate toxic metals, but some of them lacking the ability to remove toxic metals in animal tissues and inducing severe side effects made them fail to be included as an therapeutic possibility (Yan et al. 1997; Kojima et al. 1992; Tandon et al. 2003; Eybl et al. 2006; Bludovska et al. 1999). Thus, alternative approaches are needed for chelation of heavy metals in animals and humans safely and effectively.

In the recent studies, one of the possible mechanisms of heavy metal alleviation was found to be the reduced metal accumulation, and there are several strains of *Lactobacillus* proved to have the potentials (Bisanz et al. 2014; Monachese et al. 2012). Two of *Lactobacillus reuteri* strains, Cd70-13 and Pb71-1, separated by Bhakta et al. (Bhakta et al. 2012b) from sludge and mud were characterized with high sorption of metals and adhesion, which means they are not only good at uptaking metals from the environment but also good at survival in the intestinal milieu. It was the first time that one study was designed to isolate potential LAB strains that are metal-resistant and probiotic candidate in food, and it is the very first study to challenge the LAB bioaccumulation of metals from the fish intestinal milieu in vivo. LAB strains used in yogurt including one of the *Lactobacillus rhamnosus* strains, *L. rhamnosus* GR-1, can combine many toxic metals, for example, Cd and Pb, As, and Hg in vitro (Bisanz et al. 2014; Ibrahim et al. 2006). Passive sequestration was suspected to the mechanism, but alternative pathways were discovered by Bisanz et al. that enzymes play a role in detoxification of Hg by putative probiotic strains, especially in demethylation and reduction. Moreover, they also tested the preventing abilities of these food-grade microbe strains from metal uptake in the gastrointestinal (GI) tract (Monachese et al. 2012). The results were positive and give hope to a long-term intervention by probiotics on Hg and As exposure in populations that are vulnerable, such as pregnant women. This approach can be spread and benefit the people who are living near mining sites in the developed countries. More importantly, studies like this can help to build a frame in the further human trials. As reasonable as it is, the presumption of effectiveness in reducing toxin levels is the main contribution by probiotics to health benefits for children; long-term human trial studies are still needed to connect the reduced blood toxin levels to physical and cognitive development. Zhai et al. investigated the protective effects of the selected probiotic *Lactobacillus plantarum* CCFM8610 against Cd exposure and demonstrated its Cd-binding, antioxidative abilities (Zhai et al. 2015c). It was significant that CCFM8610 could provoke the excretion of Cd resulting in the increase of Cd in feces, reduced levels of Cd in hepatic, and lower Cd concentration both in acute and chronic Cd-exposed mice model (Zhai et al. 2013, 2014). There are three aspects explaining how CCFM8610 could contribute in decreasing intestinal absorption of Cd. Firstly, CCFM8610 is fast and effective in binding Cd ions; thus, along with the strains, Cd can be excreted before intestinal absorption in the feces. Secondly, as the study showed, living CCFM8610 may repair the depressed mobility of the intestine by Cd toxicity, and then, more Cd can be excreted through feces. Thirdly, as strains of *Lactobacillus* and *Bifidobacterium* can promote essential elements of absorption and bioavailability (Pérez-Conesa et al. 2006; Klobukowski

et al. 2009; Kruger et al. 2009; Scholz-Ahrens et al. 2007), Zhai et al. speculated that living CCFM8610 was firstly dedicated to the absorption of divalent essential elements instead of Cd, which subsequently limited the intestinal absorption of Cd. We have stressed the importance of Cd-binding abilities of CCFM8610, but it is also important to consider the functions of intestinal barrier which may be balanced and enhanced due to the improvement of the immune responses by probiotics (Lutgendorff et al. 2009; Bron et al. 2012; Zakostelska et al. 2011; Patel et al. 2012). Thus, further investigations were needed to perform test on CCFM8610 of whether more routes are involved in inhibiting Cd absorption in addition to Cd binding, with special attentions on the protection of the intestinal barrier. Such study have been done by Zhai et al., in which CCFM8610 were compared with another two *L. plantarum* strains, CCFM8614 and CCFM 8611, by introducing them orally into mice that have been exposed to Cd (Zhai et al. 2016a). The results showed that these three strains differ in Cd-binding ability and antioxidant ability, but they all remarkably increased the levels of Cd in feces. CCFM8614 can work on alleviated oxidative stress induced by Cd exposure in the intestines, protect the gut barrier function, decrease Cd permeation, and further inhibit Cd absorption. As to CCFM8610, it has high antioxidative activities which could then prevent the gut barrier function from Cd-induced oxidative stress and finally reduce Cd absorption by the intestine. Comparatively, CCFM has lower protective effects on the gut than CCFM8610, because it simply excretes Cd from the gut by binding. According to the results showed above, the protection of intestinal barrier is the main route of protecting the body from Cd absorption, and it cannot be understood just as simple indirect effects from the Cd-binding strains; instead it is also the result of a direct effect from the antioxidative ability of *L. plantarum* strains. Thus, the *L. plantarum* CCFM8610 is quite a candidate for application in fruit and vegetable juices due to its Cd removal ability. By administrating CCFM8610-fermented soymilk, the Cd in mice feces, livers, and kidneys was also significantly reduced (Zhai et al. 2016b). There is also one specific *L. plantarum* strain that owns the ability to alleviate the severe toxicity syndromes in mice caused by acute and chronic Al exposure by Yu et al. (Yu et al. 2016b, c). CCFM639 increased fecal Al levels and decreased Al levels in livers, kidneys, spleens, brains, as well as the blood, indicating the excretion of Al from Al-exposed mice. There are several mechanisms involved in this phenomenon. CCFM639 could act on the Al sequestration in the intestine, or the protection of intestinal barrier, or the alleviation of Al-induced inflammatory responses and oxidative stress (Yu et al. 2016a). A mice model has been established by Tian et al.to determine the role of *L. plantarum* CCFM8661 in easing the lead-induced effects (Tian et al. 2012). The Pb levels in the blood and tissues were significantly reduced by CCFM8661, especially in the intervention group compared to the therapy group. There was no obvious difference of the alleviation ability between living and dead CCFM8661 cells in both intervention and therapy experiment. This research team led by Tian et al. also performed the experiment on protecting mice from Cu toxicity using another *L. plantarum* strain, CCFM8246 (Tian et al. 2015). After the oral administration of cooper ions, the accumulations were mainly observed in the livers, kidneys, and brains of mice, which were significantly higher than that of the

control mice ($p < 0.05$). Then, strain CCFM8246 was orally administrated to Cu-exposed mice, and it decreased the Cu concentrations in all of the organs mentioned above. It was the same in the protection models, in which CCFM8246 were given before the exposure to Cu. Further interesting study would be the application of these probiotic strains in fermentation of food which would be accessible to people daily and protect them from heavy metal toxicities.

8.3.2 Recovery of Heavy Metal-Induced Dysfunction

For the past few years, industrial progress has been made along with numerous metal pollution incidents, resulting in serious public health concerns. Cd, Al, and Pb are nonessential toxic metals that have a harmful effect on the liver, kidney, brain, reproductive system, and so on (Rozman and Klaassen 2007; Nordberg et al. 2014). Cu is also a toxic metal if its level exceeds the safe limits (Ward et al. 2001; Gaetke and Chow 2003). There are several media for the general public to contact these toxic metals, such as air, water, food, etc. (Nordberg et al. 2014; Volpe et al. 2009). After oral ingestion, the metals are absorbed in the intestinal tract and then accumulated in different organs and tissues. Therefore, the very first organ that is susceptible to toxic metals is the gastrointestinal tract (Nordberg et al. 2014). Toxic metal accumulation, including Cd, Pb, Cu, and Al, can induce inflammation causing various dysfunctions in biochemical and physiological process.

Since the accumulation of Cd is mostly in mice kidney and liver, they are also the major targets of acute Cd exposure (Nordberg 2009; Jihen et al. 2010; Goyer and Clarkson 2001). Histomorphology remains a powerful routine evaluating tissue injuries in animal models. Zhai et al. (Zhai et al. 2013) reported living CCFM8610 treatment significantly alleviated hepatic injury. Synthesis of metallothionein (MT) could be induced in the liver after the absorption of Cd in the intestine. MT, which has a tendency to bind Cd, is a low-molecular-weight protein (Nordberg and Nordberg 2000). The Cd-MT complex can be stored in the liver, and it can also be transported from the bloodstream to the kidney. Oxidative stress can be induced if the binding capability of MT to Cd is saturated in the liver and kidney, the extra Cd ions would induce oxidative stress, and subsequently these tissues would be damage (Klaassen and Liu 1997; Nordberg and Nordberg 1987). However, during the chronic Cd exposure, most of the Cd is stored in the liver instead of the kidney in the beginning, and they are then transported from bloodstream to kidney (Friberg et al. 1985), the chronic Cd-induced hepatotoxicity in human is drawing more and more attention. There are enough evidence suggesting that severe illness in the liver, such as pathological and functional dysfunction, can be caused by chronic Cd exposure (Koyu et al. 2006; Uetani et al. 2006), indicating that further investigation is needed on how chronic Cd toxicity results in hepatic damage. After chronic Cd exposure, kidney was recognized as a target organ by many studies. Renal proximal tubular cell injury has been proved to be the mechanism of this nephrotoxicity

(Brzóska et al. 2003; Goyer and Clarkson 2001). Besides the damages on kidneys, tissue damage is also one of the consequences of chronic Cd exposure, especially by intraperitoneal injection (Zhai et al. 2014). In the liver, histological damages can be induced by Cd exposure. To be specific, after the chronic exposure of Cd, liver plates cannot hold to be intact, and also chromatin condensation and cytoplasmic vacuolization would be induced. The group of animal that were receiving both Cd and CCFM8610 had less abovementioned hepatic injury. The combination also alleviated kidney injuries induced by Cd exposure, such as tubular necrosis, dilation of glomeruli, and swollen glomeruli. As we have known that the inhibition of Cd absorption is partly due to the binding ability of probiotics, Zhai et al. focus on their protective roles in the gut barrier (Fig. 8.7) (Zhai et al. 2016a). They reported a reducing Cd-induced cytotoxicity, a restoring tight junction disruption, and repairing gut permeability by CCFM8610. Finally, the improved gut barrier induced the increasing Cd accumulation in fecal and correspondingly decreasing Cd levels in tissues. Besides, antioxidative ability of *L. plantarum* strains may also result in alleviation of Cd-induced gut barrier dysfunction.

Al accumulates in various mammalian tissues, including the liver, kidneys, spleen, bone, and brain (Ward et al. 2001), causing the dysfunction of hepatocellular integrity and hepatic function (Kaneko et al. 2004), nephrotoxicity (Mahieu et al. 2003), and neurotoxicity. Even some experts believe that the accumulation of Al can contribute to Alzheimer's-like neurofibrillary tangles as it can across the blood–brain barrier and then form deposits in the brain (Sharma et al. 2009). Probiotics, especially *L. plantarum* CCFM639, have been widely demonstrated to be proactive against Al-induced tissue injuries. Yu et al. demonstrated that *L. plantarum* CCFM639 is able to lighten tissue damage of the kidney and brain, especially acute

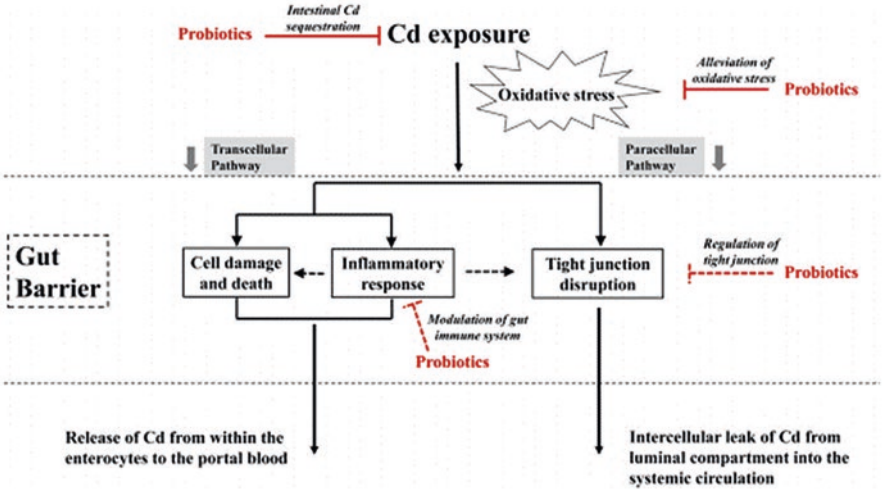

Fig. 8.7 The proposed mechanism for gut barrier disruption and potential protective pathways with probiotics under Cd exposure

Al-induced serious liver injury (Yu et al. 2016c). The increase of AST and ALT in the circulation, which is induced by chronic Al exposure, indicates the occurrence of liver and kidney injury (Andy and Keeffe 2003). Al exposure also resulted in the increase of CRE and BUN, revealing the metabolic disorder and damage to the kidneys (Mahieu et al. 2009).

The reduction of these parameters caused by *L. plantarum* CCFM639 indicated that it may also help to relieve damage in the liver and kidneys (Yu et al. 2016b). In addition, Al is a neurotoxic metal and can lead to learning and memory impairment and anxiety-like behaviors (Mathiyazahan et al. 2015; Prakash et al. 2013). *L. plantarum* CCFM639 alleviating Al-induced neuronal injury has been confirmed by Yu et al. The protective mechanism may be attributed to three routes (Fig. 8.8). Firstly, *L. plantarum* CCFM639 has strong affinity for binding Al; thus it can decrease the Al absorption in the intestine and reduce Al level in the circulation and in the brain, thereby alleviating Al-induced neurotoxicity. Secondly, CCFM639 elevated the integrity of TJs that are mainly composed of transmembrane proteins, such as occludin and claudins and ZO-1. Intact TJs can form a protective barrier to prevent harmful substances from entering the brain and maintaining CNS functions (Sampson and Mazmanian 2015). Lastly, CCFM639 can alleviate cerebral oxidative stress and decrease pro-inflammatory cytokines in the brain of mice, subsequently reducing Aβ accumulation and AChE activity in the brain. These results support the potential use of *L. plantarum* CCFM639 as a new dietary intervention against neural injuries caused by Al exposure (Yu et al. 2017).

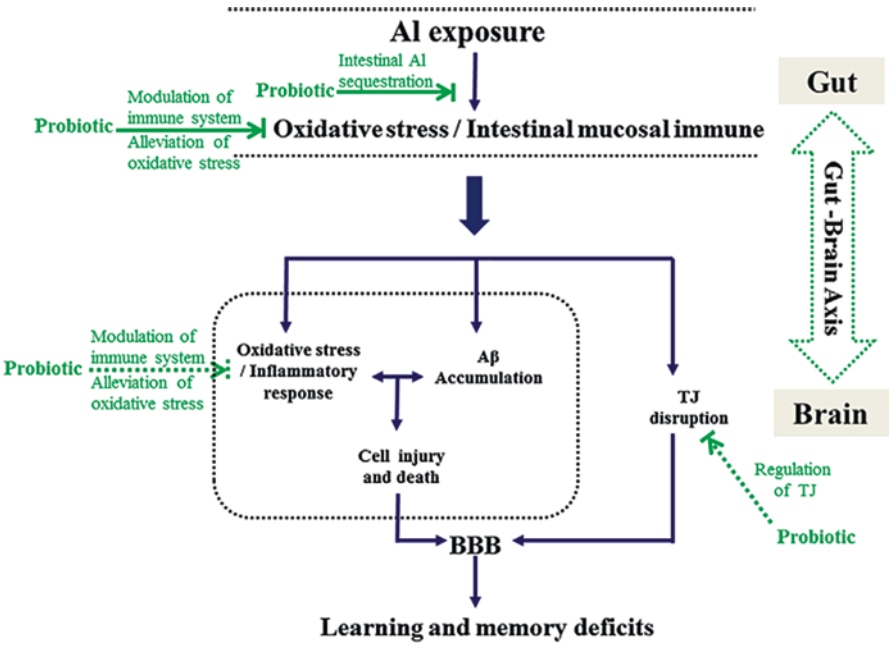

Fig. 8.8 The potential protection mechanism of probiotics against Al-induced neurotoxicity

Pb toxicity has been associated with neurologic and hematological dysfunctions, renal and hepatic damage, and reproductive disorders in human body (Solon et al. 2008). *L. plantarum* CCFM8661 can decrease the Pb levels in the blood and tissues, prevent oxidative stress induced by Pb exposure, and finally offer a significant protection against Pb toxicity (Tian et al. 2012). Therefore, the strain also has the potency to recover Pb-induced tissue dysfunction.

Cu, mainly found in the liver, also circulates with the blood and finally arrives at the brain, kidneys, and other organs (Chen et al. 2007). Excessive Cu directly damages the liver, kidneys and brain, influencing learning and memory ability, as shown in Wilson's disease (Frydman et al. 1985). The protection role of *L. plantarum* CCFM8246 in Cu toxicity has been demonstrated to be associated with reduced Cu contents in tissues, reversed oxidative stress induced by Cu exposure, as well as normalized ALT and AST levels in serum, and finally improved mice's spatial memory (Tian et al. 2015). It is worth mentioning that AST and ALT, as specific markers of hepatic function (Imperiale et al. 2000; Andy and Keeffe 2003), indicated the alleviation of liver damage by CCFM8246. As we know, it is the first research reported the protective effects of lactobacilli on excessive Cu accumulation in tissues, including improving oxidative stress in the renal and hepatic, reducing hepatic damage.

8.3.3 Alleviation of Heavy Metal-Induced Oxidative Stress

The toxicity caused by heavy metal exposure, such as Pb, Cd, and Zn, is one of the historic environmental problems, and still caused global health concerns today (Zhai et al. 2015a). The toxicity of heavy metal can be attributed to the disruption of calcium homeostasis, direct damage to the mitochondria and mitochondrial membranes, oxidative stress, and so on (Ahamed and Siddiqui 2007). Among these mechanisms, oxidative stress has been extensively studied. Heavy metal exposure has been reported to lead to excessive reactive oxygen species (ROS), damage the antioxidant defense system of cells, and increase lipid peroxidation (Farmand et al. 2005; Tian et al. 2012; Zhai et al. 2014; Yu et al. 2016c). For example, excessive Cd depleted the level of reduced glutathione (GSH), inhibited antioxidant enzymes, and strengthened production of ROS, which in turn caused elevating lipid peroxidation and oxidative DNA damage (Liu et al. 2009; Ognjanović et al. 2010; Thijssen et al. 2007).

The chelation therapies, mainly chelating heavy metals to promote their excretion, are the most direct treatment yet lack safety and efficacy validation (Yan et al. 1997; Kojima et al. 1992). Thus, new strategies are still in urgent requirement. Recently, phytochemicals and plant extracts have been widely reported for their potentials in antioxidation and thus to be able to reduce oxidative stress induced by the toxic metal and lower their levels in tissue (Vicente-Sánchez et al. 2008; Renugadevi and Prabu 2009; Jelenkovic et al. 2014; Reddy et al. 2014). With few side effects, these natural compounds can be added to diet as one of the nutritional ingredients. This leads

researchers to seek other dietary supplements to prevent the toxicity of toxic metals. The GSH system, containing synthesis, transport, uptake, and redox turnover, is completed in some *Lactobacillus* strains. Therefore, there strains, commonly used in daily life, are potential to alleviate oxidative stress (Wang et al. 2012; Kullisaar et al. 2010; Mikelsaar and Zilmer 2009). A bunch of studies demonstrated that *Lactobacillus* strains exert antioxidant ability via high-antioxidant enzyme activities, suppressed lipid-peroxidation reactions, and improved lipid metabolism (Chen et al. 2005; Zhang et al. 2010; Güven and Gülmez 2003). For example, *Lactobacillus* casei Zhang can reduce lipid peroxidation and improve lipid metabolism in the blood and liver of hyperlipidemic rat (Zhang et al. 2010). *Lactobacillus* GG can ameliorate oxidative stress in intestine caused by alcohol digestion (Forsyth et al. 2009). *Bifidobacterium catenulatum* ZYB0401 in combination with *L. fermentum* ZYL0401 can decrease the level of MDA and improve the activity of SOD in liver (Xing et al. 2006). *L. fermentum* ME-3 can lower the levels of peroxidized lipoproteins and enhance total antioxidative activity (Kullisaar et al. 2003).

MDA and ROS are, respectively, a by-product of the lipid peroxidation and a biomarker of oxidative stress. And SOD, GSH-Px, and GSH are considered to play important roles in antioxidant defense system. Depletion of GSH is reported to induce mitochondrial dysfunction and the impairment of energy metabolism, followed by an ROS accumulation (Heales et al. 1995; Pereira and Oliveira 2000). *L. plantarum* CCFM8610 with a good Cd-binding capability has been reported to protect against acute and chronic Cd-induced toxicity in mice for both the livers and kidneys (Zhai et al. 2013, 2014). They confirmed that Cd intoxication caused a markedly elevating MDA and a decreasing GSH, SOD, and CAT, while *L. plantarum* CCFM8610 treatments reversed these effects, indexes including CAT, SOD, GSH, and MDA in the liver, as well as SOD, GSH, and MDA in the kidney.

However, whether this alleviation was only caused by the initial Cd sequestration in the intestine with the addition of CCFM8610 and thus reduced Cd absorption, or related to antioxidant ability of CCFM8610 itself, remains unclear. Therefore, Zhai et al. designed an experiment in which mice were exposed to Cd via intraperitoneal injection and avoided the direct contact between Cd and CCFM8610 (Zhai et al. 2014). Due to failure of Cd sequestration effect in the intestine, the decreasing MDA levels in tissues, which belong to Cd-plus-CCM8610 IP group, were caused by alleviated oxidative stress of CCFM8610. Besides, significantly higher MT protein, an effective free radical scavenger, was observed in the Cd-plus-CCFM8610 IP group compared to the Cd-only IP group, indicating that CCFM8610 may improve the production of host MT proteins. As to tissue Cd levels, no obvious difference was found in two groups. Thus, the higher MT production induced by CCFM8610 uncovers the direct effect of CCFM8610 on oxidative stress resistance. Therefore, the proposed pathways of protection by CCFM 8610 against Cd toxicity were shown in Fig. 8.9. Another evidence include the studies on flavonoid (Vicente-Sánchez et al. 2008) and *Ganoderma lucidum* spores (Jin et al. 2013). Either of them was administrated to the rodent and increased the MT level in tissues and was demonstrated to protect against Cd-induced oxidative stress. Comparative whole transcriptomic analysis of tissues from the Cd-only and Cd-plus-CCFM8610 IP mice groups showed that co-treatment of Cd and CCFM8610 was associated with

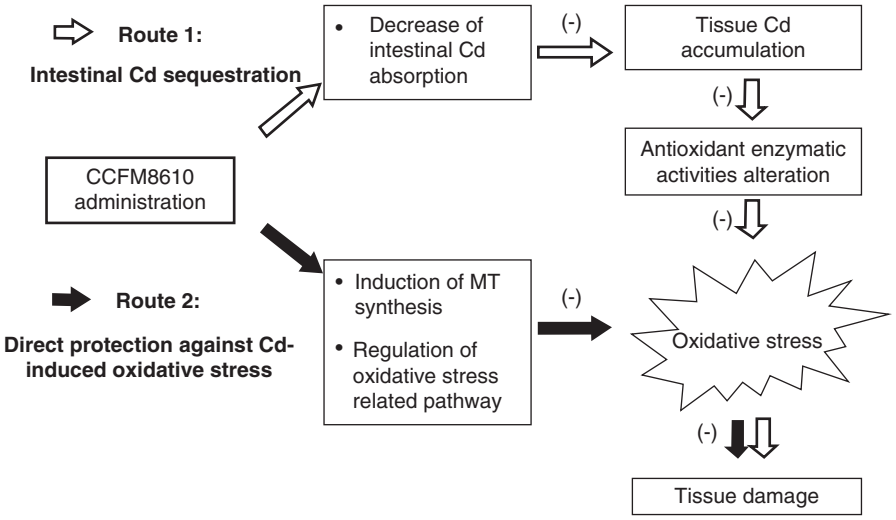

Fig. 8.9 The proposed pathways of protection by CCFM 8610 against Cd toxicity

gene expressions, which is in several pathways related to Cd toxicity. For instance, chronic Cd exposure caused upregulation of several genes involved in the mitogen-activated protein kinases (MAPK) pathways in the liver, which might relate to over-production of ROS and act on Cd-induced cellular apoptosis. Finally, they confirmed that the protection role of CCFM8610 can be not only caused by the primary Cd sequestration in the intestine but also more direct antioxidative stress capability. The inhibition of ALAD activity by Pb causes an accumulation of ALA, which further induces the generation of ROS and oxidative stress. Many reports indicate this may be an important mechanism of Pb toxicity (Monteiro et al. 1991; Hermes-Lima et al. 1991). *L. plantarum* CCFM8661 has been evaluated by Tian et al. and shows a protection against Pb-induced toxicity in mice (Tian et al. 2012). Treatment of *L. plantarum* CCFM8661 firstly recovered the ALAD activity and reduced ROS levels induced by Pb exposure. What's more, CCFM8661 can also increase GSH and SOD levels and decrease MDA and GSH-Px levels in kidney. These improvements in antioxidant enzyme activities and reductions in the levels of oxidative stress-related molecules, accompanied by restorations of deficits in antioxidant defense system, indicate a direct protective role of CCFM8661 in Pb-induced oxidative stress. The antioxidative ability might be one of the main mechanisms for *L. plantarum* CCFM8661 to alleviate Pb toxicity.

As for Cu toxicity, excessive Cu exposure has been reported to inhibit activities of SOD, GPx, and other antioxidant enzymes and depleted reduced GSH, which result in accumulated free radicals, lipid peroxidation, as well as oxidative DNA damage (Sakhaee et al. 2012; Matović et al. 2015). Tian et al. investigated the protection role of *L. plantarum* CCFM8246 against Cu intoxication in vivo (Tian et al. 2015). The results showed that mice liver, a crucial organ for Cu storage, possessed SOD and GPx with higher activities in *L. plantarum* CCFM8246 treated group

compared to the copper-only group. In addition, simultaneous decrease in MDA levels was also observed in treatment group. All of these evidences support *L. plantarum* CCFM8246 as a protective supplementation against oxidative stress induced by Cu exposure.

Al toxicity on various organs, such as the liver, kidney, and brain, is commonly considered as associated with altered activities of antioxidant enzymes and burdened free radicals (Bhadauria 2012). Both aspects disturbed the antioxidant defense system, which is believed to be the main mechanism underlying oxidative stress caused by Al exposure. Previous studies demonstrated a markedly increasing MDA level and AChE activity, as well as a decreasing GSH level and SOD, CAT, and GPx activities. However, *L. plantarum* CCFM639 co-treated with Al can influence several antioxidant defense system-related parameters: reduce lipid peroxidation; improve GPx, SOD, CAT, and AChE activities; decrease MDA level; and increase GSH level. The results demonstrate the alleviative effect of this strain on oxidative stress induced by Al exposure (Yu et al. 2016b, c, 2017). The one possible reason is that *L. plantarum* CCFM639 decreases Al absorption in intestine and reduces Al accumulation in tissues, which significantly attenuates Al-induced oxidative stress. The other possible reason is antioxidant activity of *L. plantarum* CCFM639 itself, which may be associated with exopolysaccharides (EPS) (Wang et al. 2016) or a complete GSH system. Overall, probiotics have great potential to be a therapeutic dietary strategy against oxidative stress induced by toxic metal.

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Chapter 9
Lactic Acid Bacteria and Food-Based Allergy

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Contents

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https://doi.org/10.1007/978-981-13-1559-6_9

Abstract Food allergy is an abnormal immune response to food. The symptoms and signs may range from mild to severe. The prevalence of food allergy is increasing, and consumers are now more aware of allergy and intolerance than ever. This chapter introduces the symptoms and mechanisms of food allergy and the method to prevent food allergy. Lactic acid bacteria, which are widely used in the food industry, have positive effects either on cellular immunity or humoral immunity. This chapter also introduces how mucosal vaccines for food allergy are constructed based on lactic acid bacteria.

Keywords Food allergy · Oral tolerance · Mucosal immune system · Expression system

9.1 Food Allergy

The term food allergy is "an adverse health effect arising from a specific immune response that occurs reproducible on exposure to a given food," as defined by Boyce et al. (2010). Food allergy is a global health problem that affects millions of people and multiple aspects of a person's life. The current prevalence of food allergies in different countries or regions is still lack of large-scale cross-regional epidemiological data. Due to the differences of research methods, crowd selection, and case identification standards, these food allergy rates or prevalence data cannot be compared directly. But the epidemic state about different types of food allergy or different living backgrounds in the same area has great reference, which can guide the risk assessment of food allergens, logo management, food trade, and so on. Food allergy is estimated to affect 15 million Americans, approximately 4% of children and 1% of adults, and the prevalence increases continuously in the past two decades (Gupta et al. 2011; Sicherer et al. 2010). There are increasing published reports on the prevalence of food allergy in infants in developing countries such as South Africa and China. Both countries were previously considered that food allergy seldom happened. Chen et al. recruited 497 infants and young children (0–12 months old) in Chongqing for routine well-baby checks at the Children's Hospital of Chongqing Medical University. Parents completed questionnaires, and children were skin prick tested (SPT) to ten foods in total together with histamine and saline controls. The overall prevalence of challenge-proven food allergy in children aged 0 to 1 year was 3.8% (18/477, 95% CI, 2.5–5.9%), with 2.5% (12/477) egg allergy and a 1.3% (6/477) prevalence of cow's milk allergy (Chen et al. 2011). The South African Food Sensitization and Food Allergy study (SAFFA) recruited 544 children aged 12 to 36 months from childcare education facilities in Cape Town. The prevalence of challenge-proven immunoglobulin E (IgE)-mediated allergy was 2.5% (95% CI, 1.2–3.9%), with 1.8% egg allergy, 1.2% peanut allergy, and 0.2% to cow's milk (Basera et al. 2015).

Food allergies can theoretically be caused by any food, while over 90% of food allergies are caused by eight categories of food including eggs, milk, peanuts, tree nuts, shellfish, fish, wheat, and soy (Boyce et al. 2011). The differences of dietary habits in different areas can lead to the prevalence of certain types of local food

allergy. For example, mustard, sesame, celery, lupine, and molluscan shellfish have been identified as significant allergens in European countries (Burks et al. 2012), while bird's nest allergy is the most common in Singapore (Shek and Lee 1999).

9.1.1 Symptoms and Severity

Food allergy has a wide variety of symptoms (Table 9.1). The most common symptoms include the appearance of itching of the lips or tongue, repeated vomiting, frequent diarrhea, or urticaria. However, allergic reactions show significant difference in severity. For patients with severe peanut allergy, exposure to milligram scale of peanut protein can provoke life-threatening clinical symptoms within seconds or up to 2 h (Burks 2008).

According to the time interval between consumption of foods and onset of symptoms, food allergies can be divided into two types. The first type is characterized as immediate reaction. Symptoms like anaphylaxis (shock) or urticaria occur within a few minutes or even seconds after intake of certain food. These kinds of symptoms are usually serious and generally caused by foods such as eggs, nuts, peanuts, fish, and shellfish. The second type of food allergy is late reaction, in which the relatively lighter symptoms (such as fatigue, irritability, depression, and hyperactivity) appear

Table 9.1 Symptoms associated with food allergic reactions

Cutaneous	Pruritus	**Gastrointestinal**	Oral pruritus
	Erythema/flushing		Oral angioedema (lips, tongue, or palate)
	Urticaria		Pharyngeal pruritus/tightness
	Angioedema		Colicky abdominal pain
Ocular	Pruritus		Nausea
	Tearing		Vomiting
	Conjunctival injection		Diarrhea
	Periorbital edema	**Cardiovascular**	Tachycardia
Respiratory			Dizziness
Upper	Pruritus		Loss of consciousness/fainting
	Nasal congestion		Hypotension
	Laryngeal edema	**Miscellaneous**	Metallic taste in the mouth
	Rhinorrhea		Uterine cramping/contractions
	Sneezing		Sense of impending doom
	Hoarseness		
Lower	Cough		
	Wheezing		
	Dyspnea		
	Chest tightness/pain		

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a few hours or even several days after ingestion of the food. Thus, it is difficult to tell the accurate cause of food allergies because of the delay. Foods that can cause this type of reactions include chocolate, milk, citrus, legumes, and food additives (Zukiewicz-Sobczak et al. 2013).

9.1.2 The Mechanism of Allergic Reactions

Adverse food reactions represent any abnormal clinical response on exposure to a given food. Based on the pathophysiological mechanism of the reactions, they are further classified as food intolerance or food allergy. Food intolerance is an adverse physiologic response and may be caused by the inherent feature of the food (pharmacologic active component, toxic contaminant, etc.). These kinds of response are often dose dependent and may not be reproducible. Food allergy is defined as an abnormal immunologic response that occurs reproducibly associated with exposure to a given food (Boyce et al. 2010). According to the different pathogeneses, food allergy can be further divided in three types. Type I is IgE-mediated, which accounts for the majority of the entire food reaction process and is the best-characterized food allergy reaction. Type II hypersensitivity is immune cell-mediated, which is caused by antibodies to cell surface antigens and components of the extracellular matrix. These antibodies can sensitize the cells for antibody-dependent cytotoxic attack or for complement-mediated lysis. Type III is mixed IgE-mediated and cell-mediated when both IgE and immune cells are involved in the reaction (Fig. 9.1).

IgE-mediated classic food allergy is diagnosed by detection of food-specific IgE. It includes the sensitization phase, excitation phase, and effector phase of the food allergen. The gastrointestinal mucosal surface encounters enormous quantities of antigen on a daily basis. Food proteins are taken up by M cells, which are specialized epithelial cells. Then they are transferred to macrophages or dendritic cells, which act as antigen-presenting cells and processed into peptide fragments presented on the cell surface by Class II MHC molecules. Peptides are then presented to naïve T helper (Th) cells via MHC/T cell receptor interaction, resulting in Th cell priming and activation (Burks 2008) (Fig. 9.2). In normal people, this process can form immune tolerance. While in the genetic predisposition of individuals, this event initiates humoral and cellular events associated with food allergy. Here peanut allergy is showed as an example. The activated T helper cells secrete cytokines to stimulate B cells to synthesize peanut-specific IgE antibodies. IgE antibodies are bound primarily on the surface of mast cells or basophils by the high affinity surface IgE receptors (FcεRI); then the body is in a sensitized phase. During the excitation phase, after ingestion, the peanut protein is cross-linked with the specific IgE antibodies on sensitized mast cells and basophils. The cells then release a large number of bioactive medium including histamines, leukotrienes, prostaglandins, and platelet-activating factors. At the effector phase, these molecules act on the various tissue organs and lead to all kinds of food allergy symptoms (Hodge et al. 2009).

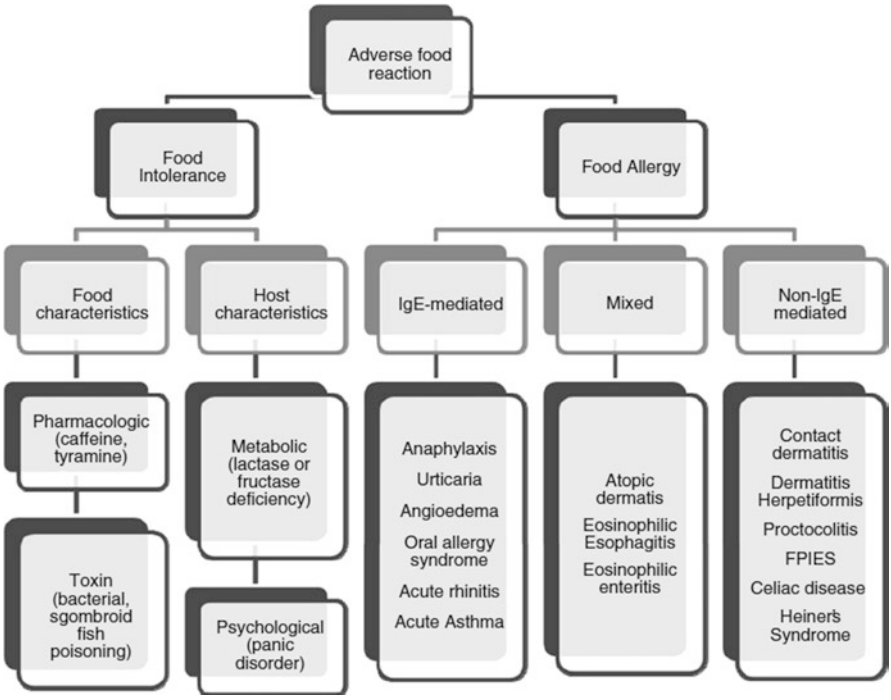

Fig. 9.1 Classification of adverse reactions to foods. (Originally printed by Antonella Cianferoni (Cianferoni and Spergel 2009). Reproduced with permission from Elsevier Limited)

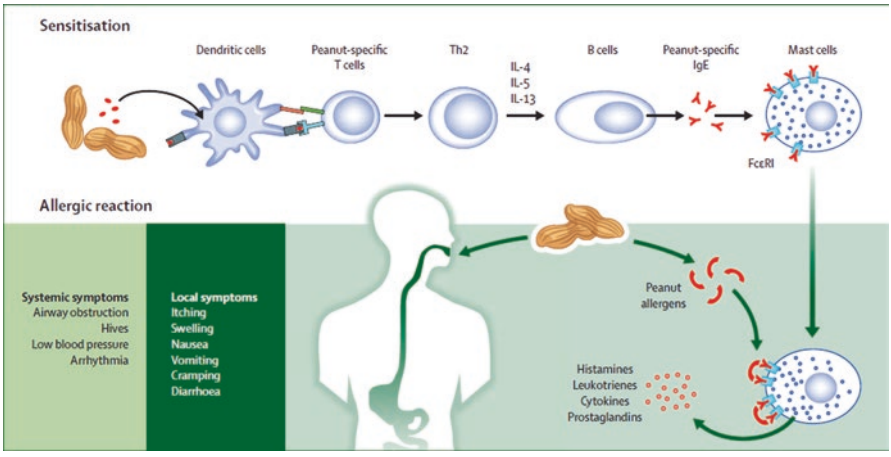

Fig. 9.2 Peanut allergy. (Originally printed by Burks (2008). Reproduced with permission from Elsevier Limited)

Non-IgE-mediated food allergy reaction involves type II and III hypersensitivity, which is mainly caused through the release of Th2 cytokines and the lack of the cytokines that can regulate T cells. Non-IgE-mediated food allergies are lack of significant features. They may be aroused by the additives or natural ingredients of food and often expressed as acute or chronic gastroenteritis. In the pathogenesis of non-IgE-mediated food allergy, eosinophils and T cells mainly participate in cellular immune response. Inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin 13 (IL-13), and IL-5 also play important roles. Non-IgE-mediated mechanisms of food allergies are lack of sufficient evidence, but there are some studies (Yan and Shaffer 2009; Mills and Breiteneder 2005; Sicherer and Sampson 2006; Sicherer 2002). For example, milk-induced thrombocytopenia belongs to type II hypersensitivity, and food-induced malabsorption mainly relates to type III food allergy. Known mechanisms of non-IgE-mediated food allergy include Th1-mediated responses, immune-complex formation leading to complement activation, or T-cell/mast cell/nerve cell responses, including the functional changes of smooth muscle and small bowel motility. The most study of non-IgE-mediated food allergy is celiac disease. This disorder is due to alcohol-soluble prion-stimulated T cell immune response, and deamination of alcohol-soluble prion will strengthen this kind of immune response. Some chemical histamine-release agents and histamine-containing foods (chocolate, tomato, and strawberry) can directly act on the mast cells and cause allergic reactions. In addition, the inherent characteristics of food allergens may be the factor of food allergy. Common food allergens are water-soluble glycoprotein, and the relative molecular mass is between 10×10^3–70×10^3. They are also resistant to heat, acid, and protease. These properties protect the stability of food allergens (Shreffler et al. 2006). In general, IgE-mediated food allergy and non-IgE-mediated responses are not mutually exclusive.

9.1.3 Oral Tolerance and the Prevention of Food Allergy

Sometimes the body produces no or low-specific immune responses after ingesting a certain food antigen but still has normal immune reaction to other antigens. This phenomenon is called oral tolerance. It can be said that oral tolerance is an immunosuppressive state of ingesting antigens through oral pathways (Van and Knippels 2007). Failure in inducing oral tolerance can lead to the occurrence of food allergy. Oral tolerance was first reported by Wells and Osborne in 1911. They fed the ovalbumin (OVA) antigen to animals first; the allergic reactions could be reduced when OVA was injected again. In addition, intestinal epithelial cells, dendritic cells, and T regulatory cells play important roles in inducing oral tolerance. Intestinal epithelial cells act as non-specific antigen-presenting cells. Dendritic cells secrete IL-4, IL-10, and regulatory T cells and produce TGF-β, which are beneficial to the occurrence of oral tolerance. In recent years, oral tolerance has been successfully used in autoimmune disease model. It also shows certain application prospects in the treatment of human autoimmune diseases.

The mechanisms of oral tolerance include clonal deletion, clonal anergy, active suppression, and bystander suppression (Faria and Weiner 2005). For clonal deletion, the antigen in the systemic circulation can mediate the clonal removal of antigen-specific lymphocytes. The antigen can be detected in the blood after 1 h of oral ingestion, which may be one of the mechanisms of high-dose oral antigen-mediated immune tolerance. Clonal anergy is defined as T cells in a nonresponsive state, showing no proliferation of T lymphocytes and no secretion of IL-2. But the inactive state of cloned allergic cells can be reversed when they are cultured with IL-2 together. Active suppression is a process in which the activation of self-reactive lymphocytes is inhibited by other potentially inhibitory lymphocytes. Oral low-dose antigens can induce systemic no response or hyporesponsiveness by active suppression. The bystander suppression can be delimited that the activated regulatory T cells in GALT by oral antigens migrate into the body's lymphoid organs and directly suppress the production of the effector cells. Or they migrate to specific organs and release non-specific cytokines such as IL-4, IL-10, and TGF-β to inhibit Th1 cell activation, so as to prevent the development of pathogenesis of immune response. Recently it has been found that the generation of oral tolerance is dose dependent. High-dose-dependent tolerance is mediated by two kinds of mechanisms: Fas molecule-mediated apoptosis signal and the loss of colony-stimulating signal. Decreased activities lead to prolonged stay of T cells in the site where immune tolerance is induced. The decreased activity of T cell may relate to the expression defects of adhesion molecules and cytokine receptors on the surface. In addition, Th3 cells, Th1 cells, $CD4^+CD25^+$ regulatory cells, and $CD8^+$ inhibitory T cells are involved in oral tolerance induced by low-dose immune tolerance.

Induction of oral tolerance can be used as a potential immunotherapy for the treatment of food allergy (Garside and Mowat 2015). Over the past decade, oral immune tolerance treatment studies have shown that most patients can successfully desensitize during 1–2 years of treatment (Wood 2016). The Learning Early About Peanut Allergy (LEAP) trial and follow-up studies tested the hypothesis that regular consumption of peanut-containing products, when started during infancy, would elicit a protective immune response rather than an allergic immune reaction. In brief, the study randomly assigned 640 children aged 4–11 months with high risk for peanut allergy to consume peanuts or avoid them until 5 years of age. Children in the consumption group ate a designed food-containing peanut at least three times a week. Among the 530 infants who initially had a negative peanut skin test result, the prevalence of peanut allergy at 5 years age was only 1.9% among children who ate peanuts, as compared with 13.7% among those who avoided peanuts. Among the 98 babies who initially had a positive skin test result, the prevalence of peanut was 10.6% in the consumption group and 35.3% in the avoidance group. Overall, sustained consumption of peanuts beginning in the first 11 months of life was highly effective in preventing the development of peanut allergy. Based on these data, the authors recommend the introduction of peanuts in the first 4–6 months of life significantly decreased the frequency of peanut allergy in this exceptionally high-risk population (Du Toit et al. 2015).

In addition to the concept of early introduction, there is great interest in other approaches to prevention. The composition of the gastrointestinal microbiota has

been postulated to play a role in the development of allergies because it can suppress Th2-induced allergic inflammation. From this perspective, the use of probiotic supplementation seems an attractive option for the prevention and treatment of allergic diseases. Cuello-Garcia et al. performed a systematic review of randomized trials focused on probiotics and allergy prevention (Cuello-Garcia et al. 2015a). They concluded that probiotics can reduce the risk of eczema when used by women during the last trimester of pregnancy (relative risk [RR], 0.71; 95% CI, 0.60–0.84), when used by breastfeeding mothers (RR, 0.57; 95% CI, 0.47–0.69) or when given to infants (RR, 0.80; 95% CI, 0.68–0.94).

However, there are still some problems in clinical application of oral tolerance. For example, it is difficult to grasp the dose of oral antigen. Low dose is ineffective, while high dose can aggravate the condition. Although a single large dose of antigen ingestion can significantly reduce the incidence of acute autoimmune disease and improve clinical symptoms, multiple oral antigens must be taken to achieve a significant effect for recurrent autoimmune disease. In addition, allergy relapses once stop taking antigen. These restrict the oral tolerance toward to the clinical treatment of food allergies.

9.2 Lactic Acid Bacteria and Immune System

9.2.1 Mucosal Immune System

The mucosa covers the largest surface area of the human body, including the airway, oral cavity, digestive tracts, ocular cavity, and genitourinary tract, and it provides the interface for interactions between the interior body and its external environment. Because of its large surface area and continuous exposure to the outside world, the mucosa is the primary entry route of numerous pathogens and microbes (Lamichhane et al. 2014). Therefore, these mucosal sites form a defense system, mucosal immune system, to prevent the occurrence of local mucosal disease and to maintain immunological homeostasis through innate and acquired immunity.

The mucosal immune system is the primary site to perform local-specific immune function. It is mainly composed of gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), conjunctiva-associated lymphoid tissue (CALT), and urogenital tract mucosa-associated lymphoid tissue (UALT). According to the different distributions and functions of mucosa, the mucosal immune system can be divided into inductive sites and effector sites (Kiyono and Azegami 2015). The inductive site is the first place where antigens are contacted and ingested, such as GALT and BALT. GALTs are equipped with most of the immunocompetent cells required for the generation of antigen-specific humoral and cell-mediated immune responses (Zuercher et al. 2002) (Kunisawa et al. 2012), including organized follicular structures such as Peyer's patches (PPs), cryptopatches, and isolated lymphoid follicles (ILFs). PPs are considered to be one of the largest organized lymphoid tissues in the gastrointestinal immune system. PPs contain efferent but not afferent lymphatics. To compensate, PPs are covered with the

follicle-associated epithelium containing specialized antigen-sampling membranous cells (M cells). M cells act as professional antigen sampling and gateway cells for the mucosal immune system, as they allow the selective and efficient transfer of antigens from the intestinal lumen into PPs. Orally administered antigens are taken up by M cells and then promptly deliver to antigen-presenting cells, such as dendritic cells (DCs) and macrophages. DCs are abundant in the subepithelial dome region under the follicle-associated epithelium. They can immediately process and present orally encountered antigens from M cells to mucosal T cells, which clusters of B cells and T cells lie next to M cells in the epithelium (Yamanaka et al. 2001). B cells, a major component of PP cells (~75%), are induced and switched from IgM to IgA. They are preferentially located in the follicle region. As a result, PPs contain B cells at several differentiation and maturation stages: IgM^+B220^+(~70%), $IgM^+IgA^+B220^+$(~1%), IgA^+B220^+(~3%), and IgA^+B220^-(~0.5%) (Kunisawa et al. 2012). Approximately 20% of PP cells are T cells. Most of them are naïve T cells. The active phenotypes of T cells include IFN-γ-producing Th1, IL-4-producing Th2, and IL-10-producing Foxp3+ regulatory T cells (McGhee et al. 1989). Concurrent with antigen presentation, DCs located in PPs upregulate gut-imprinting molecules (e.g., CC chemokine receptor 7 (CCR7), CCR9, CCR10) on antigen-specific lymphocytes through the retinoic acid cascade for their subsequent migration to the effector tissues (Sato and Iwasaki 2005; Yuki and Kiyono 2009).

At the effector sites (e.g., intestinal lamina propria), the differentiation of IgA^+B cells is induced by IgA-enhancing cytokines (such as IL-5, IL-6, and IL-10). The terminal products are plasma cells that produce dimeric or polymeric forms of IgA (Kunisawa et al. 2012; Mestecky and McGhee 1987; Cerutti 2008). These IgA antibodies then bind to polymeric immunoglobulin receptors located on the basal membrane of epithelial cells and are transported to gut secretions as the form of SIgA (Mostov et al. 1984). This collaborative and well-orchestrated sequence between the inductive (e.g., PPs) and effector (e.g., intestinal lamina propria) sites provides the immunologic basis for the induction and regulation of antigen-specific immune responses (e.g., SIgA production) at the mucosal surface (Fig. 9.3).

9.2.2 Effects of LAB on the Immune Response

Lactic acid bacteria (LAB) constitute a phylogenetically related group of anaerobic Gram-positive bacteria, which share a fermentative metabolism and convert sugars into lactic acid. The original habitat of many LAB species is unclear, but some of them are separated from the gastrointestinal microflora of vertebrates. In general, LABs are known as safe and beneficial for their use in the preparation of fermented foods such as pickles, sausages, cheese, and dairy products. Another advantage of LAB is that they can stimulate both innate and adaptive immune response by increasing macrophage activation and antibody production (Haller et al. 2000). Certain obligate anaerobic bacteria like *Bifidobacterium* and *Lactobacillus* combine with specific receptor in intestinal epithelial surface, forming fixed bacterial membrane and biological barrier, which can effectively resist the invasion of pathogens.

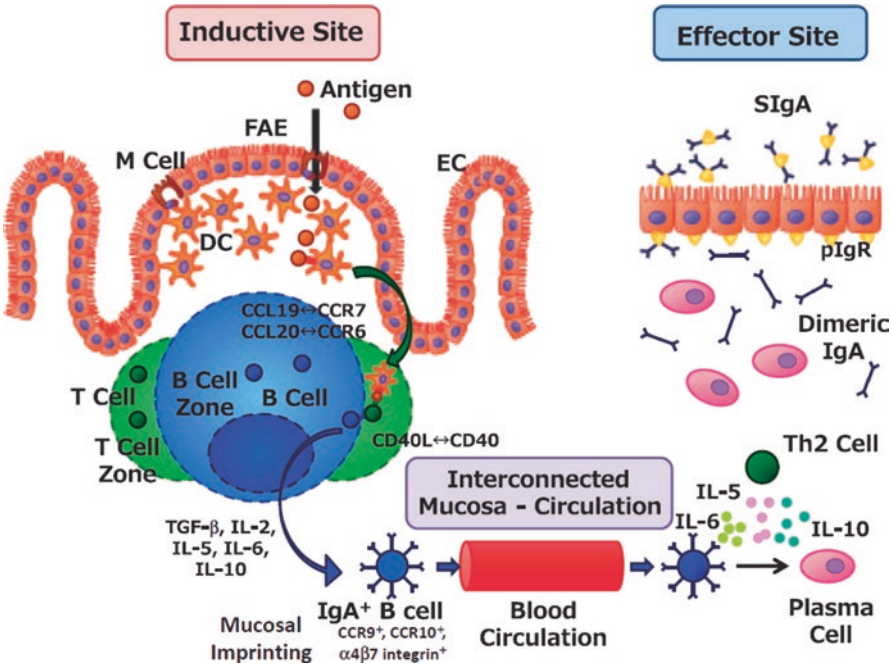

Fig. 9.3 Coordination between inductive and effector sites for the induction and regulation of antigen-specific mucosal immune responses. (Originally printed by Kiyono and Azegami (2015). Reproduced with permission from Elsevier Limited)

Studies also have found that LAB can firm intestinal mucosal barrier, improving immune adjuvant performance. Candela reported that *L. casei*, *L. rhamnosus*, and *L. plantarum* can enhance systemic and mucosal immunity (Candela et al. 2008). *L. acidophilus* can adhere to intestinal epithelial cells (Hao et al. 2015). *B. animalis* Bb12 can effectively activate phagocytic cells (Holscher et al. 2012a, b). The function of LAB on mucosal immunity mainly reflects in the following aspects.

9.2.2.1 LABs Recognize Intestinal Mucosal Cell Receptors

Pattern recognition receptor (PRR) is a type of protein that play a crucial role in the proper function of the innate immune system. Some LAB can be combined with PRR on the surface of intestinal epithelial cells, thus triggering innate immunity and adaptive immunity. The pathway of PRR signaling affects the activation of antigen-presenting cells (APCs) such as DCs and the polarization of T cells. *L. rhamnosus* can stimulate the phagocytic receptor on the intestinal phagocytic membrane (FPRs), activate NOX2, and induce intestinal phagocytic cells to produce ROS, thus repairing the injured mucosal immune system (Alam et al. 2014). IL-6 production is inhibited when viable or nonviable cultures of *L. casei* CRL431 interacted with intestinal epithelial cells previously treated with anti-mouse TLR2 antibody, which

suggests that *L. casei* CRL431-IEC interaction is mediated completely through TLR2 (Vinderola et al. 2005). The fact that particles from probiotic bacteria can interact with the intestinal immune cells suggests that TLR-9 could also be involved in the signal process to activate the immune cells of the gut (Dogi et al. 2010).

9.2.2.2 LAB Regulate sIgA Secretion

LAB can increase the numbers of IgA-secreting cells in mouse's LP. Localized sIgA on the intestinal mucosa surface can neutralize toxins and prevent invasion by infectious agents (Wittig and Zeitz 2003). In addition, intestinal mucosal epithelial cells can produce secretory component, combined with the two-body IgA molecules to form sIgA that can prevent intestinal protease decomposition. LAB can improve the response capability of T and B lymphocyte cells against antigens and, meanwhile, can activate the relevant lymphoid tissue in the intestinal mucosa. Perdigon et al. (1995) reported that *L. casei* and *L. acidophilus* might increase the production of sIgA and the number of antibody-forming cells in the small intestine of mice. *L. casei* increased the total sIgA level and did not produce the specific sIgA induced by probiotics. IgA and IL-6 levels produced by the lamina propria were significantly increased in mice fed with *L. casei* ($P < 0.05$). The absence of specific antibodies to *L. casei* demonstrated that the beneficial bacteria of the intestinal immune system were unresponsive (Galdeano and Perdigón 2006). In addition, peptides released by *L. helveticus* during milk fermentation can also increase the response to sIgA (Leblanc et al. 2004). Treating rats with EHEC prior to treatment with this bioactive peptide showed an increase in IgA and sIgA levels in the intestinal lamina propria. Infants intake of *Bifidobacterium* can effectively stimulate cell proliferation of IgA secretion and improve the immunity of infants. It has also been reported that adding *B. Bb12* to infant diet for 6 weeks can increase the levels of endocrine anti-poliomyelitis and anti-rotavirus IgA.

9.2.2.3 Effects of LAB on Intestinal Mucosal Immune Cells

LAB can activate B and T lymphocytes in intestinal mucosal and enhance the immune response. LAB can also stimulate the macrophages in the intestinal mucosa, make them swallow, kill and digest a variety of pathogenic microorganisms. When the old men took LAB for 4 weeks, proportion of B lymphocyte subsets were significantly increased (Perdigón et al. 2001). Furthermore, LAB can improve IgA, IgM, and IgG levels in mucosal surface and serum by itself or cell wall components that enhance humoral immunity and promote proliferation of T and B lymphocytes. It is also proved that LAB can enhance cellular immunity and vitality of mononuclear phagocytic cells (monocytes and macrophages) and polymorphonuclear leukocytes, which strengthen non-specific immune response. Extracellular polysaccharide (EPS) of LAB is an immunomodulator, which can activate the immune receptor, and improve immune function. Besides, different LABs have

various effects on the maturation of DCs, which affect the association between NK/DC cells. Majority of LAB and plasma cell-like dendritic cells can stimulate cytotoxic potential of NK cells and promote antiviral innate immune response (Rizzello et al. 2011). High doses of *L. rhamnosus* Lcr35 can promote human monocyte-derived dendritic cell maturation in an in vitro experiment (Evrard et al. 2011).

LAB has got more and more attention from researchers, and a mass of beneficial LAB products and functional foods continue to emerge. Daily diet with supplement of LAB can adjust intestinal flora, improve the intestinal environment, inhibit the growth of harmful microorganisms, and activate mucosal immunity. Over the past few decades, although LABs in the intestinal immune regulation already have lots of studies both at home and abroad; the basic theoretical research about the impact of LAB on intestinal mucosal is still modest. Mounting researches focused on the exploitation of high-quality probiotics and new strains as well as industrial applications. To better understand the relationship between LAB and intestinal immunity, as well as relationship between intestinal microbial community and human health, we should not only focus on the development of new strains but also pay attention to the mechanism of LAB in the intestine adhesion colonization, stimulation, and the production of immune response cells.

9.3 Lactic Acid Bacteria and Allergy

The continuous increasing allergic diseases lay a huge financial burden on health care due to potentially life-threatening allergic reactions, associated direct and indirect costs, and reduced quality of life (Asher et al. 2012). The composition of the gastrointestinal microbiota has been considered to play an important role in the development of allergies. Studies showed that gastrointestinal microflora can promote potentially antiallergenic processes, Th1-type immunity, generation of tumor growth factor (TGF, which has an essential role in suppression of Th2-induced allergic inflammation and induction of oral tolerance), and IgA production, an essential component of mucosal immune defense (Prokesova et al. 2006; Cuello-Garcia et al. 2015b). These results suggest that alterations of gut microbiota, the early and most massive source of microbial exposure, might resolve the allergic epidemic. From this perspective, oral ingestion of probiotics, defined as a live microbial feed supplement that beneficially affects the host by improving the intestinal microbial balance, seems an attractive way for the prevention and treatment of allergic diseases. Lactic acid bacteria are probiotics that can act as promoters to balance in the gut microbiota, which in turn could delay or prevent the development of allergic diseases. The preventative effects of probiotics to eczema, the early manifestations of allergic disease, have been evaluated in many randomized controlled trials. Most studies used single strains of lactobacilli and bifidobacteria or their combination (Boyle et al. 2011; Dotterud et al. 2010; Rautava et al. 2012; Wickens et al. 2008).

Cuello-Garcia et al. reported in their meta-analysis that there was a benefit of probiotics for eczema reduction when administered in the last trimester of pregnancy or during breastfeeding when administered to infants and/or mothers (Cuello-Garcia et al. 2015b). The effects of probiotics on food and respiratory allergies were also evaluated by researchers (Canani et al. 2017; Canani et al. 2016; Cabana et al. 2017). A pooled analysis included 17 trials (2947 infants) in a meta-analysis showed that the combination of prenatally and/or postnatally probiotic treatment decreases the risk of atopy and food sensitization in young children (RR 0.78; 95% CI 0.66–0.92) (Zhang et al. 2016). This was most evident when probiotics were given to pregnant mother and postnatally to child (RR 0.71; 95% CI 0.57–0.89; 1–2 = 0%). A combined prenatal plus postnatal probiotic administration also reduced the risk of food hypersensitivity (RR 0.77; 95% CI 0.61–0.98; 1–2 = 0%).

Researches on the anti-allergic mechanism of lactic acid bacteria mainly focus on their effects on the production of Th1 and Th2 cytokines in the body, including IL-4, IL-5, IL-10, IL-13, IFN-γ, IFN-α, TNF-α, etc. It is considered that the positive effects of lactic acid bacteria on allergic diseases mainly owe to two aspects, that is, the regulation of cellular immunity and the regulation of humoral immunity.

9.3.1 Regulation of Cellular Immunity

The stimulation of foreign antigen raises a series of cellular immune responses. Lactic acid bacteria can interfere with the cellular immunity. The activation of cellular immunity results in the activation of macrophages and B lymphocyte nuclear NK cells and the production of cytokines such as interleukin and interferon (Ghadimi et al. 2010; Schabussova et al. 2011). A comparison of the immunoregulatory responses of four *Lactobacillus* (*Lactobacillus casei*, *L. rhamnosus*, *L. plantarum*, *L. reuteri*) and two strains of *Bifidobacteria* found that they all enhanced the proportion of $CD69^+$ on lymphocytes, T cells, T lymphocyte subsets, and nature killer (NK) cells. At the same time, the levels of cytokines such as IL-1β, IL-6, IL-10, and TNF-α were increased to a certain extent. *Lactobacillus* strains tended to promote Th1 cytokines, whereas bifidobacterial strains were likely to produce a more anti-inflammatory profile (Dong et al. 2012). Oral administration of lactic acid bacteria also have protective immunomodulatory effects on allergic airway inflammation mice induced by house dust mites. *Streptococcus thermophilus* CCFM218 (ST218) could strongly induce IL-10 production and significantly suppress IL-4 secretion in vitro. The in vivo anti-allergic property of ST218 was then evaluated in a mouse allergy model together with *L. rhamnosus* GG. ST218, which had a better suppressive effect on allergic response in vivo, characterized by increased specific IgG2a and IL-10 levels in serum, regulatory T cells in the mesenteric lymph nodes, and a reduction in serum Th2 cytokine IL-4 (Ai et al. 2015).

Many studies have shown that the anti-allergic ability of lactic acid bacteria was strain specific. This kind of ability can be realized by either live or dead cells. The immunomodulatory capacity of *Leuconostoc citreum* L3C1E7, a strain isolated from Pico cheese, was assessed by Domingos-Lopes et al. In mice fed with OVA, plasma quantity of allergen-specific IgG1 and IgG2a was significantly raised, while plasma levels of these antibodies were significantly reduced in mice co-feeding with the strain. Moreover, plasma OVA-specific IgE level was significantly decreased by ingestion of the strain. These results showed that administration of L3C1E7 could suppress the synthesis of allergen-specific IgE and might improve Th2-mediated allergic symptoms (Domingos-Lopes et al. 2017). *Bifidobacteria* can improve gastrointestinal symptoms and promote the maturation and development of immune function in very low birth weight infants. Similarly, bifidobacteria can effectively modulate immune cell activity in patients with specific reactive dermatitis, improve helper T cell and T cell activity, and thus slow the symptoms of specific reactive dermatitis (Drago and Iemoli 2014).

9.3.2 Regulation of Humoral Immunity

It is proved that lactic acid bacteria possess the functions of adaptive immunity related with activation of lymphocytes and stimulation of antibody production. Humoral immunity is a kind of immune responses that plays a major role with specific antibodies. With the different types, the way into the body, and the immune response process of pathogens, the immune system can synthesize and secrete five kinds of immunoglobulin antibodies: IgG, IgA, IgM, IgE, and IgD. These antibodies can recognize specifically bound antigens, mediate immune cell activity, and enhance the phagocytic function of phagocytes.

Secretory immunoglobulin IgA is the main antibody against local mucosal anti-infective immunity and mainly distributes in the saliva, tear, gastrointestinal fluid, milk, and respiratory secretions. As the main immunoglobulin in intestinal mucosa, it is the first line of defense against various endogenous commensal bacteria and exogenous pathogens. *Bifidobacteria* can stimulate the body to produce IgA cell proliferation and increase IgA levels. Orally administrated *Bifidobacterium animalis* Bb12 to infants for 6 weeks stimulates higher levels of IgA production, which can play a significant role in enhancing immune cell activity and immunoglobulin levels (Holscher et al. 2012a, b). Intake of probiotic Dahi restrained the raise of whey–protein-specific IgE and IgG response in whey–protein-sensitized mice. Moreover, sIgA levels in intestinal fluid collected from mice administered with probiotic Dahi were significantly increased (Shandilya et al. 2016).

IgG accounts for more than 75% of the total antibodies in serum, which is the most important antibody component in serum and extracellular fluid. When feeding of probiotic *L. rhamnosus*-fermented milk either to suckling mothers or to their

offspring, there was a significant decrease in physical symptoms of allergy. Considerable reducibility of OVA-specific antibodies (such as IgE, IgG and IgG1) and ratios of IgE/IgG2a and IgG1/IgG2a in the sera of neonatal mice were recorded (Saliganti et al. 2015). Intravaginal inoculation of *L. reuteri* CRL 1324 to mice showed an immunomodulatory effect on the cells and mediators of innate immunity, decreasing the number of neutrophils induced by the pathogen maternal group B streptococcus (GBS) and increasing the activated macrophage population. Moreover, mice inoculated with *L. reuteri* CRL1324 were observed with an increase in B lymphocytes and IgA and IgG subclasses (De Gregorio et al. 2016).

IgE is the main antibody that causes type I hypersensitivity. It is synthesized by B cells in the lymphoid tissues of the respiratory tract and gastrointestinal mucosa. IgE is distributed in the mucosal cells, exocrine fluid, and blood of these sites, which is the mediator of allergic reactions. The antigenicity and IgE-binding capacity of four major proteins in *L. casei*-fermented milk were reduced by 15–90% relative to the original feedstock (Shi et al. 2014). Studies have demonstrated that *L. delbrueckii* subsp. b*ulgaricus* CRL656 can hydrolyze the three major BLG epitopes and decrease their recognition by IgE of allergic patients (Pescuma et al. 2011).

9.4 Mucosal Vaccines for Food Allergy

LABs are Gram-positive and generally recognized as safe. Recombinant LAB strains were proved to elicit specific systemic and mucosal immune responses against selected antigens effectively in mice model. For this reason, this group of bacteria is considered as promising candidates to produce heterologous proteins and as a potential replacement of classical microbial carriers. *Lactococcus lactis* is the mainly used LAB in genetic engineering for heterologous protein production. *L. lactis* has been used in the food industry through history, particularly in the production of fermented foods. These food-grade bacteria are non-colonizing and do not produce lipopolysaccharides or other toxins. Besides, the genomes of several *L. lactis* subspecies have entirely sequenced, and numerous expression systems have been successfully designed.

9.4.1 Expression Systems of L. lactis

As *L. lactis* has become the paradigm of LAB, the essential features of the expression systems, their development, and their applications are mainly focused on *L. lactis*. Various gene expression systems have been developed for *L. lactis* to produce heterologous proteins.

9.4.1.1 Constitutive Promoters

Promoters that are not known to be controlled by any regulator or growth conditions are presumed to be constitutive under laboratory growth conditions. Five promoters—P21, P23, P32, P44, and P59—are commonly used as a constitutive lactococcal promoter (van der Vossen et al. 1987), which means that the heterologous proteins can be continuously produced by the engineered host. Strong (P21, P23, and P59) and weak (P32 and P44) promoters were distinguished by the chloramphenicol acetyltransferase activity levels. Numerous heterologous proteins, such as M6 from *Streptococcus pyogenes*, were directed by P23 and P59 promoters in *L. lactis* IL1403 (Piard et al. 1997), and phenylalanine ammonia-lyase from *Petroselinum crispum* was produced by P32 promoter in *L. lactis* MG1363 (Xiang et al. 1999).

9.4.1.2 Inducible Promoters

Inducible promoters generally drive the expression of genes involved in cell adaptation to its environment. In most cases, inducible promoters are preferred over constitutive promoters because they can be exactly controlled by the user. Various lactococcal inducible systems are available now, such as sugar-inducible expression system (Miyoshi et al. 2004), lytic phage attack system (OSullivan et al. 1996), chloride-inducible gene expression cassette (Sanders et al. 1997), temperature, or pH shift (Miyoshi et al. 2004). However, without doubt, the most widely used and successful lactococcal expression system to date is the nisin-inducible controlled gene expression (NICE) system developed by Kuipers and his colleagues in 1995 (Kuipers et al. 1995). Nisin is a 34-amino acid bacteriocin produced by *L. lactis* and is encoded by a cluster of 11 genes. NICE comprises the regulatory elements of the *nis* operon: P_{nisA}, the nisin-inducible promoter, and *nisRK*, the regulator–sensor two-component system. The host *L. lactis* NZ9000 is derivative from the nisin-negative MG1363 strain integrated with the *nisRK* genes into the chromosome. When the targeted gene is placed under the control of the P_{nisA} promoter on the expression plasmid such as pNZ8048, transcription of that gene can be induced in the presence of subinhibitory amounts of nisin (Kuipers et al. 1998). This system offers numerous advantages, such as easy to use, tightly controlled, and efficiently induced, large-scale production process. However, nisin addition remains costly for industrial production.

9.4.1.3 Protein Secretion in *L. lactis*

Secretion is mostly generally preferred compared to cytoplasmic production in the engineering of heterologous protein expression, due to the advantages such as higher yields, simpler purification process, and better target interactions (Le Loir et al. 2005). *L. lactis* only secretes one major protein of 45 kDa (Usp45), whose

function is still unknown (van Asseldonk et al. 1990). Besides, the selected laboratory strains do not produce any extracellular protease, so that the secreted proteins have less prone to be extracellularly degraded (Lee et al. 2000). Furthermore, *L. lactis* possesses only a unique exported housekeeping protease, *HtrA*, which can degrade improperly folded proteins. *HtrA* knockout *L. lactis* strains are available now, and these mutant strains allow for higher heterologous protein secretion because their degradation is impaired (Miyoshi et al. 2002).

9.4.1.4 Protein Surface Displayed on *L. lactis*

A monolayer membrane envelope and the relatively thick cell wall of *L. lactis* permit them suitable for anchoring bioactive molecules to the bacterial surface. Targeted covalent molecule binding to an anchor domain and non-covalent binding are the two main ways to anchor recombinant proteins to the cell surface. There are five different types of anchor domains which have been detected and applied in lactic acid bacteria. They are the transmembrane anchors, lipoprotein anchors, LPXTG-type cell wall anchors, Acma-repeat anchors, and S-layer protein attachments (Song et al. 2017). The most commonly used anchoring unit is the LPXTG-type cell wall anchor. This type of cell wall anchor contains an LPXTG sequence motif (where X is any amino acid) followed closely by a hydrophobic transmembrane domain and a short positively charged tail. Secretion signal guides the proteins which export across the cell membrane via the Sec-pathway. Upon secretion, a conserved catalytic transpeptidase called sortase cleaves the LPXTG motif of the polypeptide between the threonine (Thr) and glycine (Gly) residues. The protein is then covalently attached to the cell wall peptidoglycan via the Thr (Mazmanian et al. 1999). Several examples successfully displayed heterologous proteins through sortase-mediated cell wall anchoring way (Kobierecka et al. 2016; Christophe et al. 2015; Kajikawa et al. 2011).

9.4.2 *Engineered* L. lactis *for the Production Heterologous Proteins*

L. lactis is a strictly homolactic fermentative bacteria, which convert the carbon source completely into lactate. Lactic acid is an industrially important ingredient. It is used as an acidifier for preservation, as a flavor development material in the food production, and as an important raw compound in the pharmaceutical industry (Papagianni 2012). Although *L. lactis* is not known as a high-efficiency secretion host, it simultaneously secretes and/or anchors heterologous proteins such as enzymes and therapeutic molecules to make them interesting candidates for such purpose. *L. lactis* can obtain amylolytic capacity through secreting amylases. For example, Okano et al. (2007) genetically modified *L. lactis* IL1403 secreting

α-amylase from *Streptococcus bovis* 148, which has been known to degrade raw starch efficiently. This recombinant strain was capable of fermenting soluble starch, although its efficiency was relatively low (0.09 g/L/h). By using *L. lactis* cells adapted to maltose instead of glucose, a more rapid starch fermentation was accomplished (1.31 g/L/h). Maximum volumetric lactate productivity of 1.57 g/L/h was further achieved by using cells adapted to starch.

9.4.3 Engineered L. lactis *as Vaccine Delivery System*

Vaccination is the most effective method to prevent and control infectious diseases. Vaccines play a profound role in the improvement of human and animal health. An ideal mucosal vaccine should stimulate an effective humoral and cell-mediated immunity response to a specific antigen, provide a long-lasting protection after a single dose in early infancy, and be stable and nontoxic. Vaccine development relies on live viral or bacterial vectors, enabling antigen production in vivo. Attenuated strains of pathogenic species (mainly belonging to the genera *Salmonella*, *Shigella*, *Yersinia*, and *Listeria*) are often used as tools for non-parenteral vaccination (Detmer and Glenting 2006). However, there is attendant risk of such constructs reverting to a virulent phenotype. When it comes to vaccine design, the employing of *L. lactis* as vaccine delivery vehicles unveils exciting new possibilities for their advantages of oral administration and mucosal immune stimulation (Zhang et al. 2011; Ai et al. 2016).

Tetanus toxin, a model antigen from *Clostridium tetani*, has been expressed in *L. lactis* to develop pioneering vaccines. It was targeted to different locations of strains in comparative studies of strains. Steidler et al. constructed a constitutive expression strain of *L. lactis* producing the tetanus toxin fragment C (TTFC) within the cytoplasmic compartment. When mice were intranasally administered to the bacteria, the level of anti-TTFC antibody increased rapidly and was substantially higher in mice immunized with IL-2 and IL-6 adjuvants (Steidler et al. 1998). Since then, a variety of antigens against either animal or human diseases have been produced, secreted, or surface anchored in *L. lactis*, such as human papillomavirus-16 (HPV), hepatitis E virus antigen (HEV-Ag) (Gao et al. 2015), *Staphylococcus aureus* HtrA protease (Samazan et al. 2015), etc.

Peanut allergy, an IgE-mediated hypersensitivity disease, is one of the most common and severe food-induced anaphylaxes. At present, strict avoidance of the peanut as allergen is the only effective management strategy. Yet it is difficult to implement owing to the ubiquitous distribution of peanut ingredients in food and possible cross- or post-contamination during food processing. As a result, accidental ingestion of peanut-containing food is unusual and accounts for severe consequence each year. For allergic diseases, it is a promising immunotherapeutic strategy to induce antigen-specific oral tolerance designed to reestablish the homeostasis of the immune system. Conventional allergen desensitization strategy depends on ingestion of whole peanut extract or through parenteral routes (subcutaneous injec-

tion). However, this approach has high risk of potential safety issues due to the complex components. Genetically recombinant purified allergen proteins may decrease the risk and induce effective oral tolerance (Huibregtse et al. 2007), while the need of purification and frequent immunization might limit clinic applications of this therapy (Srivastava et al. 2002). Ren et al. (2014) employed *L. lactis* to produce recombinant peanut allergen Arah2 via different protein-targeting systems, which could be administrated through mucosal routes. The coding sequence of Arah2, the most important peanut allergen, was first optimized without signal peptide for both *E. coli* and *L. lactis* codon usage. Then resulting nucleotide sequences were synthesized and named as rA. Three different expression vectors pNZ1, pNZ2 and pNZ3 based on plasmid pNZ8148 were constructed to target rA. Recombinant strains were obtained by electro-transformation with the vectors. After being induced with nisin, these recombinant *L. lactis* could successfully produce rA protein in cytoplasm, culture supernatant, and surface of the cell. A murine model of PNA was established to evaluate the immunomodulatory potency for allergic immune responses of the three mucosal *L. lactis* vaccines. Oral administration of recombinant LAB producing the rA protein prior to sensitization could provoke effective modulatory functions both at the local sites and the systemic levels. The results demonstrated the exploitability of *L. lactis* as mucosal vaccine vectors to perform regulatory roles in peanut-allergic immune responses. Compared to the intracellular form, the forms of secretion and anchored to the cell wall were more effective in shifting the allergic immune responses from a Th2-polarized to a Th1-/Th2-balanced profile.

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Chapter 10
Lactic Acid Bacteria and Biotoxins

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Contents

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https://doi.org/10.1007/978-981-13-1559-6_10

Abstract Biological toxins are toxic chemicals from biological sources and have toxic effects or cause diseases and death. In order to reduce the biotoxins in food, many methods have been tried. The physical methods mainly include physical adsorption, strengthening the cleaning of raw materials, and clarification. The chemical methods mainly include additives or chemical fungicides to reduce the amount of biotoxins. However, these traditional strategies have many limitations, such as the change of flavor, the destruction of nutrition, and the influence of food functional properties. Lactic acid bacteria (LAB) have lots of potential applications as one of the biological antagonists. This kind of microorganisms has been extensively used in the fermented foods and the commensal microflora in the gut. LAB can generate some antagonistic compounds to inhibit the growth of pathogenic bacteria and restrain the undesirable spoilage microflora and then to reduce the biotoxins.

Keywords LAB · Biotoxins · Adsorption · Degradation

10.1 Biotoxins and the Hazards to Human Health

10.1.1 Introduction

Biological toxins, also known as natural toxins, refer to the toxic chemicals from biological sources or the products of plants, animals, and microorganisms, which have the toxic effects or cause diseases and death on other biological species (Zhang et al. 2014). Due to the diversity and complexity of biological toxins, many biotoxins have not been recognized. Therefore, the treatment of biological toxins and pollution prevention are still the worldwide problems.

10.1.2 Aflatoxin and the Harm on Human Health

Mycotoxins, as common biotoxin, are secondary metabolites produced by some fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium* (Terzi et al. 2014; Hathout and Aly 2014). Among these mycotoxins, aflatoxins are the most toxic because the synthesis of mRNA and DNA is disturbed, thereby leading to systemic damage to organism on account of the distractions of cellular protein synthesis (Ahlberg et al. 2015; Bren et al. 2007). Aflatoxins are usually produced by *Aspergillus flavus* and *Aspergillus parasitic* and severely damage human health due to its strong toxicity (Shetty et al. 2007). High intakes of aflatoxins can lead to the death of people and animals, and long-term consumption of food contaminated by aflatoxins can cause chronic liver, kidney, lung, and other organ lesions and even liver cancer (Bovo et al. 2014; Blagojev et al. 2012).

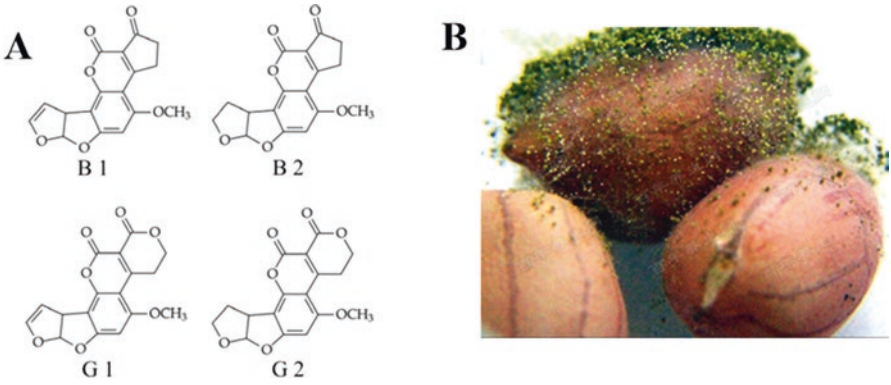

Fig. 10.1 Chemical structures of aflatoxin B1, B2, G1, and G2 (**a**) and peanut contaminated by *Aspergillus flavus* (**b**)

Aflatoxins are a class of compounds with similar structures of dihydrofuranocumarin ramification. Over 20 kinds of aflatoxins have been identified, but in feed only aflatoxin Bl, B2, Gl, and G2 are found (El Khoury et al. 2011; Barros et al. 2006). The chemical structures of these four aflatoxins are shown in Fig. 10.1. The naming of these mycotoxins is mainly based on their issued different colors under ultraviolet light. Aflatoxin B1 and aflatoxin B2 emit blue fluorescent, while aflatoxin G1 and aflatoxin G2 emit green fluorescent (Rawal et al. 2010). When poultry and livestock eat the feed contaminated by aflatoxin B1 and aflatoxin B2, these toxins will be converted to aflatoxin M1 and aflatoxin M2, most of which excreted out of the body from milk and urine but some are still residual in meat (Hell et al. 2010). Among these different aflatoxins, aflatoxin B1 is the most toxic and widely exists in food (El Khoury et al. 2011). In addition, aflatoxin B1 is the derivative of dihydrofuranocumarin and is most carcinogenic of known chemicals. The toxicity of aflatoxin B1 is mainly to the livers of humans and animals (Ahlberg et al. 2015). Except aflatoxin B1, the order for acute toxicities of the various aflatoxins is G1 > B2 > G2, while M1 and M2 are less potent than their precursors (Adebo et al. 2017).

Aflatoxins usually appear in foods on account of raw material contaminations during harvesting, storage, or processing (Williams et al. 2004). Peanut, maize, rice, wheat, and other cereals were easy to contaminate aflatoxin, which is the most toxic and harmful to humans and certain farm animal health (Hernandez-Mendoza et al. 2009b). Exposure to aflatoxins increases the risks of bacterial and parasitic infections. Aflatoxins were the extreme poison; the mortality rate of acute aflatoxicosis is up to approx. 25% in severe hepatotoxicity cases (Hernandez-Mendoza et al. 2010). While hepatocellular carcinoma or gastric carcinoma was regarded to have something with chronic exposure to aflatoxins (Ahlberg et al. 2015), aflatoxins also have impacts on immunological system, and nutritional absorption, and then threaten human and animal health (Strosnider et al. 2006).

10.1.3 Patulin and the Harm on Human Health

Patulin (4-hydroxy-4H-furo[3,2c] pyran-2[6H]-one) is a water-soluble unsaturated lactone (Fig. 10.2) and was first isolated from *Penicillium griseofulvum* and *Penicillium expansum* by Birkinshaw in 1943. Patulin was recognized as secondary metabolites produced by some fungi species such as *Aspergillus*, *Penicillium*, and *Byssochlamys* during their growth on the fruits, like apple, peaches, apricots, grapes, and pears, especially on rotten parts of these fruits (Barad et al. 2016; Morales et al. 2010; Sant'Ana et al. 2008). The fungi species were related to the fruit rot (Fig. 10.2b).

Patulin was harmful to human health, inducing a number of toxic effects on several organs including the kidney, liver, intestinal tissues, immune system, and brain (Barad et al. 2016; Puel et al. 2010). Patulin showed obvious electrophilic reactivity, and the covalent adducts were formed when patulin interacted with electrophilic chemicals. There was a strong affinity for sulfhydryl groups in patulin, and glutathione was the main cellular target of patulin (Boussabbeh et al. 2016). The disadvantages of patulin to human health included nausea, ulceration, lung congestion, convulsions, epithelial cell degeneration, and carcinogenic, genotoxic, teratogenic, immunotoxic, and immunosuppressive effects (Boussabbeh et al. 2016; de Melo et al. 2012; Glaser and Stopper 2012; Assuncao et al. 2016). The toxic signs of patulin were agitation, in some cases pulmonary congestion, convulsions, dyspnea, edema, and ulceration, hyperemia, and distension of the gastrointestinal tract (Liu et al. 2006).

Patulin was recognized to mainly induce gastrointestinal disorders with ulceration, distension, and bleeding and even alteration in renal function at higher doses. The gastrointestinal tract was involved in the transport and metabolism of different endogenous and exogenous compounds. The intestinal mucosa was the first biological barrier encountered by natural biotoxins and was able to possibly be exposed to large amounts of dietary mycotoxins. The intestinal epithelial cell model, Caco-2, was used to evaluate the effects of patulin on barrier function of the gut mucosa. The peripheral blood mononuclear cells and blood monocyte-derived dendritic cells from human were used to scrutinize the immunomodulatory effects. When the concentration of patulin was above 12 μM, the viability of Caco-2 cell was reduced, and the integrity of Caco-2 cell monolayer was affected by exposure of patulin. The reduction in barrier function was caused by patulin with the level of zonula occludens-1 and phosphorylation of myosin light chain 2 perturbation (Assuncao et al. 2016).

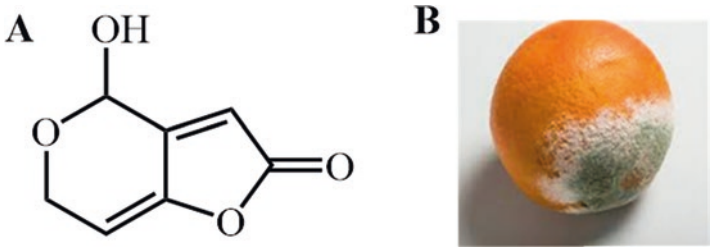

Fig. 10.2 The structure of patulin (**a**) and an orange contaminated by *Penicillium expansum* (**b**)

Table 10.1 The maximum permitted levels of patulin for fruit and their products

Country	Product classification	Limited level (μg/kg)
Russia	Fruit and fruit juices	50
	Vegetable	50
Sweden	Fruit and fruit juices	50
	Vegetable and their product	50
Switzerland	Fruit juices	50
China	Fruit and their product	50
	Semimanufactured product	100
Italy	Fruit juices	50
England	Apple juice	50
Germany	Apple juice	50

In addition, some studies had demonstrated that patulin could act as a clastogen in mammalian cells, such as micronuclei without kinetochores. It was also reported that patulin could damage the synthesis of DNA. The genotoxic influence could be involved in its capacity of reacting with sulfhydryl groups and inducing the oxidative damage. The cell membrane could be damaged by patulin through ROS generation (Liu et al. 2007). Patulin adducts with –SH group were less toxic than the unmodified compound. A quick depletion of GSH could be induced by patulin intoxication in cultured cells.

Due to the cytotoxicity, genotoxicity, and immunosuppressive properties of patulin, the European Union has established a standard for the highest permitted levels of patulin in fruit and their products (Table 10.1). The maximum level of patulin was 50 μg/kg for fruit juice and 10 μg/kg for puree-based foods for babies and young infants. In addition, Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO) have set up a provisional maximum tolerable daily intake for patulin of 0.4 μg/(kg BW)/d (Hawar et al. 2013). Patulin is thought of a moderately toxic mycotoxin for adults but exhibits remarkable toxicity for young children, and the permitted amount of patulin in food products had been regulated in several countries (Puel et al. 2010).

10.1.4 Ochratoxin and the Harm on Human Health

Ochratoxins are a group of mycotoxins produced by different molds, such as *Aspergillus ochraceus*, *Aspergillus niger*, and *Penicillium verrucosum* (Malir et al. 2016). The main forms are ochratoxin A, B, C, and D with different structures. Ochratoxin B (OTB) is a non-chlorinated form of ochratoxin A (OTA). Ochratoxin C (OTC) is an ethyl ester of OTA, and ochratoxin D (OTD) is a 4-hydroxyl of OTA (Table 10.2). In addition, OTA is the most prevalent member of ochratoxin family, while OTB and OTC are generally assumed to be less important (Heussner and Bingle 2015).

Table 10.2 Overview of main ochratoxin forms

Name	OTA	OTB	OTC	OTD
CAS number	303-47-9	4825-86-9	6529-84-6	
Molecular formula	$C_{20}H_{18}ClNO_6$	$C_{20}H_{19}NO_6$	$C_{22}H_{22}ClNO_6$	$C_{20}H_{18}ClNO_7$
Molar mass	403.8	369.4	431.9	419.8

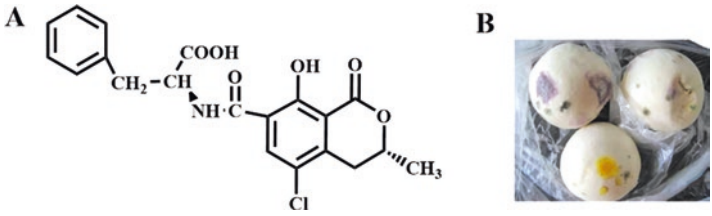

Fig. 10.3 The structure of OTA (**a**) and the steamed bud contaminated by *Aspergillus ochraceus* (**b**)

OTA is commonly contaminated in an abundance of food and animal feed and is often found in all kinds of cereals and cereal products, especially in grapes, soy, coffee, nuts, cacao, raisins, pulses, wine, spices, liquorice, and beer (Bellver Soto et al. 2014; Streit et al. 2012; Duarte et al. 2012; Iqbal et al. 2016).

The OTA toxicity is due to its chemical structure with a chlorine atom and a phenol group (Fig. 10.3), as well as a molecule of isocumarin (Petzinger and Ziegler 2000; Heussner and Bingle 2015). OTA owns a strong affinity to proteins, especially to the serum albumin promoting the bioaccumulation in the organs of animals, resulting in carry-over of the contamination (Duarte et al. 2012). Thus, muscle and offal products, milk, and eggs were easy to be contaminated, and the highest levels of OTA could be found in "black pudding," porcine blood-based sausages, and liver products (Russo et al. 2016).

Progressive renal disease, which is an endemic nephropathy, occurs in humans, which is characterized by tubular atrophy, cellular interstitial fibrosis, and karyomegaly predominately in proximal convoluted tubules (Hmaissia Khlifa et al. 2012; Grollman and Jelakovic 2007; Koszegi and Poor 2016; Petzinger and Ziegler 2000). The etiology of progressive renal disease is yet not clear, while based on the epidemiological features, researchers agree that the causative agent is of natural origin (Pepeljnjak and Klaric 2010; Woo and El-Nezami 2016). There are some hypotheses about the causes of progressive renal disease, and the two most vital hypotheses focus on the aristolochic acid from the *Aristolochia clematitis* and mycotoxins such as OTA and citrinin.

10.1.5 Microcystin-LR and the Harm on Human Health

Microcystin-LR (MC-LR) is one of the most toxic microcystins, which is harmful to the health of humans and animal (Martins et al. 2017). The stability and biological lifetime of microcystins (MCs) are due to the strong amide bonds in the cyclic heptapeptide unit (Fig. 10.4) (Karthikeyan et al. 2016).

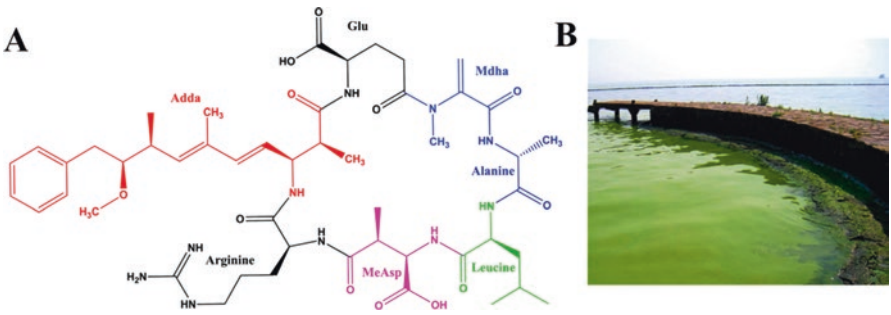

Fig. 10.4 The structure of MC-LR (**a**) and the pond contaminated by cyanobacteria (**b**)

MCs exist in rivers, lakes, and ponds, which are always used as water sources for people and livestock. The contaminated water and fishery products are the two approaches of MCs. The most serious accident involved in MCs happened at a hospital in Caruaru, Brazil, which leads to contracted toxic hepatitis of 126 patients from contaminated hemodialysis water and at least 43 died (Azevedo et al. 2002). Because of the irreversible and serious influences of MCs on the liver, traditional therapeutic strategies for MCs had little significance (Dawson 1998). Therefore, MCs, particularly MC-LR, are a non-negligible threat to the health of humans and livestock (Fischer et al. 2005). It was reported that the liver is the main target of MC-LR, which leads to serious hepatotoxicity by inhibiting specific protein phosphatases and depleting antioxidant substances (Nishiwaki-Matsushima et al. 1992). Furthermore, other harmful influences of MC-LR had been demonstrated containing reproductive toxicity in mice, liver tumor progression, and intestinal disease (Wu et al. 2014; Botha et al. 2004; Zhou et al. 2012). Oxidative damage is the key influence of MC-LR-exposed toxicities (Ding and Ong 2003). MC-LR can decrease the level of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) in the liver and increase the malondialdehyde (MDA) level in mice model, leading to the damage of antioxidant system (Xu et al. 2007; Prieto et al. 2008).

10.1.6 Other Biotoxins

Besides the above biotoxins, the major agriculturally important mycotoxins also include fumonisins, zearalenone, and trichothecenes (Hathout and Aly 2014). Fumonisins are a class of non-fluorescent mycotoxins which are produced mainly through *Fusarium verticillioides* and *F. proliferatum*, and FB_1, FB_2, and FB_3 are the major entities (Richard et al. 2003). The fumonisins inhibit ceramide synthase, inducing an adverse effect on the sphinganine/sphingosine ratio (Soriano et al. 2005). The amines of FB_1 played an important role in fumonisin toxicity (Hathout and Aly 2014). Zearalenone is a nonsteroidal estrogenic mycotoxin biosynthesized by a polyketide pathway by a variety of *Fusarium* fungi, and it is a regular contaminant of cereal crops (Zinedine et al. 2007). Trichothecenes caused apoptosis in hemopoietic progenitor cells and immune cells, and they restrained the synthesis of protein, DNA, and RNA (Richard 2007).

10.2 The Methods of Removing Biotoxins

10.2.1 Introduction

In order to reduce the biotoxins in food, many methods had been tried. The traditional methods could be summarized as physical and chemical methods. The physical methods mainly included physical adsorption, strengthening the cleaning of raw materials, and clarification. The chemical methods mainly included additives or chemical fungicides to reduce the amount of biotoxins. In recent years, many other new methods have been used to remove biotoxins.

10.2.2 Traditional Methods of Removing Biotoxins

Traditional physical and chemical methods were used to prevent fungal growth and eliminate toxin production. Water washing and organic solvent extraction can remove aflatoxins, but this will cause a loss of large amount of soluble nutrients and need the dehydration treatment, which also consume a lot of energy (Kabak et al. 2006). Moreover, organic solvent extraction can cause the residues of organic solvents in the feed and is poisonous on livestock (Rustom 1997). Water washing can cause fungal reproduction in feed if the dehydration is not timely and then lead to mycotoxin pollution again.

Some adsorbents can bind and remove mycotoxins (Kabak et al. 2006). Activated carbon has a certain adsorption ability due to its high specific surface area and porous structure. Activated carbon has a strong adsorption capacity to nonpolar organic compounds and can absorb a variety of toxins such as aflatoxin (Jindal et al. 1994). However, the activated carbon will absorb not only toxin but also nutrients with a poor selectivity (Mishra and Das 2003). Thus, activated carbon will lost its adsorption capacity to toxins after saturation of nutrient adsorption. At the same time, the feed adding activated carbon appears darkening, which affects the value of the goods and increases the cost of feed if the high-priced activated carbon is applied.

High-temperature treatment has a certain inactivation effect on mycotoxins, but most of the mycotoxins have stronger heat resistance, and it is difficult for the general heat treatment to destroy them (Scott 1984). Furthermore, excessive heat will cause Maillard reaction and other adverse reactions, leading to the loss of nutrients in feed and excessive consumption of energy (Samarajeewa et al. 1990). Therefore, it is difficult to use heat treatment in the scale feed production. Ultraviolet (UV) irradiation can inactivate the pure aflatoxin solution (Aziz and Smyk 2002). The inactivation of mycotoxins by UV irradiation is dependent on the degree of the radiation dose, the type of food, and mycotoxins (Samarajeewa et al. 1990). In addition, water plays a critical role in the destruction of aflatoxin by UV irradiation,

since radiolysis of water initiates free radical reactions leading to the aflatoxin degradation (Rustom 1997).

The addition of chemical substances (e.g., ammonia, oxidizing agent) can also remove mycotoxins, and most studies focus on the ammoniation (Mishra and Das 2003). During the ammoniation, the molecule of aflatoxin was chemically modified to new compounds with decreased or non-detectable toxic and mutagenic potentials (Park 2002). However, the added chemical substances may react with the nutrient, decreasing the nutrient value of the feed and even producing toxic or harmful substances in the feed residue. In addition, ammoniation of feed will have bad effects on the color, smell, and palatability of feed and reduce the commodity value. Moreover, ozone treatment is also applied in the removal of mycotoxins in feed (MAEBA et al. 1988). Ozone has a strong oxidation effects and can damage the reducing nutrients, such as vitamin A, vitamin E, and vitamin C, during the feed oxidation.

In order to eliminate patulin, several physical and chemical strategies have been developed to decrease the patulin concentration in food such as clarification, filtration, washing, ionizing radiation, and chemical addition. Because ascorbic acid was generally recognized as safe, inexpensive, and friendly to the sensory properties of fruit juices, ascorbic acid was applied for reducing the levels of patulin. Patulin can be degraded at all temperatures between 25 °C and 85 °C, and the degradation could be significantly increased with the addition of ascorbic acid (Kokkinidou et al. 2014).

Thermal treatment cannot completely eliminate OTA. The presence of 50% water enhanced the decomposition of OTA at 100 °C and 150 °C. Nevertheless, the complete destruction of OTA within the limits was not obtained (Boudra et al. 1995). In addition, the gamma radiation could detoxify OTA by 51.3–96.2% at a dose of 6.0 kGy (Aziz and Moussa 2004).

For OTA detoxification with chemical compounds, many adsorbent materials had been tested including charcoal, cholestyramine, sodium and calcium aluminum silicates, bentonite, wood fragment (Savino et al. 2007), and activated charcoal. The OTA concentration in a Semillon wine which contained 56 mg grape-derived protein/L and ca. 8 mug OTA/kg was reduced by 67% with the addition of bentonite at 2.5 g/L. The concentration of OTA in red wines was reduced using oakwood fragments, and the effectiveness depended on the quantity of the oakwood fragments (chips and powder), and the best results were obtained with powder (Savino et al. 2007).

MC-LR caused an oxidative damage to humans and animals, and the balance between the antioxidant and the oxidant is usually relied on endogenous protective substances and enzymes (Prior et al. 2005). Nowadays, dietary antioxidant supplements were used to help in keeping the antioxidant system. Previous studies focused on preventing or decreasing MC-LR-induced toxicity through physiological antioxidants and found that some phytochemical substances were effective in the prevention of MC-LR-induced hepatotoxicity and oxidative damage (Jayaraj et al. 2007; Sun et al. 2011; Xu et al. 2007)

10.2.3 New Methods of Removing Biotoxins

A certain amount of biotoxins in foods and their products could be removed by the traditional approaches. However, the processing of fruits couldn't ensure the complete removal of biotoxins. In addition, most of the traditional methods were expensive, inefficient, and environmentally unfriendly.

10.2.3.1 New Methods of Removing Aflatoxins

Although lots of traditional physical and chemical detoxification strategies for removing aflatoxins have been performed, the results showed that none of them really fulfills the necessary efficacy and safety (Zhao et al. 2011). As cost-effective strategies to detoxify aflatoxin-contaminated acquired and foods are imminently needed to reduce the potential losses to the farmers and the toxicological danger to the consumers, it is necessary to find new and suitable strategies for the aflatoxin decontamination (Hathout and Aly 2014). The most promising alternative for aflatoxin elimination may be through microbial detoxification. The development of microbial detoxification measures is important to ensure the safety of human foods (Samuel et al. 2014). Aflatoxin degradation by microorganisms is making use of the microbial catabolic pathways to detoxify the aflatoxins to less toxic intermediates or end products (Samuel et al. 2013). Microbial degradation owns many advantages, for example, the product specificity, mild reaction conditions, and feasible processes, when used in the food and feed industries (Kolosova and Stroka 2011).

Yeast has been applied in food processing and preservation (Hathout and Aly 2014). Yeast cell wall components, such as the polysaccharide, protein, and lipids, have the special structures, which produce the adsorption force to toxins through hydrogen bonds, ionic bonds, and hydrophobic force (Shetty et al. 2007). The aflatoxin binding by yeast cell walls is due to the mannan-oligosaccharides, which consist primarily of phosphorylated glucomannans and are designed to influence microbial ecology (Gonçalves et al. 2015). Previous researches have indicated that mannose oligosaccharides can be combined with aflatoxins to eliminate the effects of toxins on the body, and the efficiency depends on the pH, toxin concentration, and the amount of mannose oligosaccharides (Raju and Devegowda 2000). The enzymes from mycotoxin-degrading microbes are also directly applied to removing aflatoxins (Yehia 2014; Wu et al. 2015). But in practical applications, the enzyme is not heat-resistant and easy to be inactivated in the high-temperature process of pellet feed. In addition, the high cost of enzyme detoxification method makes it difficult to popularize in practice.

Because of the short degradation time and non-pigmentation in food, the strategies of microbial degradation are preferred in the food and feed industries (Teniola et al. 2005). As some fungal species can produce aflatoxins, certain species had been reported to be able to degrade aflatoxins (Shcherbakova et al. 2015; Das et al. 2014; El-Shiekh et al. 2007). The fungal metabolites could reduce the medium pH,

and the acidic condition could decrease the aflatoxin concentration (Wu et al. 2009). A class of microorganisms had been identified which own the genes coding for enzymes degrading aflatoxins, such as laccases, oxidases, and peroxidases (Shcherbakova et al. 2015). The percentage degradation of aflatoxins by culture filtrates of *Pleurotus ostreatus*, *Peniophora* spp., *Bjerkandera adusta*, and *Phanerochaete chrysosporium* can be up to 36%, 52%, 28%, and 14%, respectively, which simultaneously resulted in a loss of fluorescence and mutagenicity (Alberts et al. 2009). Indeed, these cultures were reported to exhibit laccase activities.

In recent years, scientific reports had shown lots of bacteria have the capacity of degrading aflatoxins (Sun et al. 2015; Eshelli et al. 2015; Sangare et al. 2014). Among all the bacteria which were used to detoxify aflatoxins, lactic acid bacteria (LAB) are most widely used (Adebo et al. 2017). This class of microorganisms has been proved to own a good potential in eliminating aflatoxins and can be applied as additives in food processing (Shetty and Jespersen 2006). Antifungal compounds LAB produced are supposed to decrease the production of the toxin and play a key role in the control of aflatoxin synthesis (Gourama and Bullerman 1995). The antifungal compounds usually include organic acids, phenolic compounds, hydroxyl fatty acids, hydrogen peroxide, reuterin, and proteinaceous compounds. In particular, the low-molecular-weight metabolites which were produced at LAB early growth stage have a remarkable inhibitory activity against the aflatoxin accumulation (Dalié et al. 2010). Furthermore, owing to the ability of binding to mycotoxins in LAB strains, they are considered as potential novel biological strategies to decrease the mycotoxins toxicity or protect the human body against the mycotoxin absorption (Ahlberg et al. 2015). The abilities of LABs to detoxify aflatoxins are due to their high affinity to the toxins through a binding procedure (Hathout et al. 2011).

10.2.3.2 New Methods of Removing Patulin

Nowadays, new materials based on biopolymers have been studied in the adsorption process for removal of patulin. Biopolymers could be used to reduce the level of patulin in fruit juice through adsorption. The cross-linked xanthated chitosan resin (CXCR) was prepared by using carbon disulfide as modification agent and glutaraldehyde as cross-linker. Peng et al. had investigated the biosorption of CXCR for patulin from fruit juice, and the biosorption was influenced by pH, temperature, contact time, and initial concentration. The optimum adsorption conditions of CXCR for patulin were obtained at pH 4.0 and 30 °C for 18 h, and the maximum adsorption capacity was 130 mg/g. It seems that the cross-linked xanthated chitosan resin is an effective biosorbent for reducing patulin levels in fruit juice (Peng et al. 2016). In addition, magnetic chitosan combining Fe_3O_4 particles and chitosan using Triton X-100 were selected to perform the adsorption behavior assay of patulin.

Molecularly imprinted polymer (MIP) material was a kind of synthetic macromolecular materials which contained shape-specific molecular recognition cavities from the template molecule imprint. The created specific recognition sites were

selectively extracted by the specific materials from complex matrices. MIPs have a high sensitivity, great selectivity, and fast response; thus they are used efficiently for environmental contamination, food safety, and public health.

MIPs could be one method for patulin elimination from fruit juice. The uptake capacity of patulin by MIPs was 1.55 mmol/g, and the patulin adsorption on MIP reached a stable state in just 20 min (Anene et al. 2016). In addition, the target molecule-binding sites could be located in the inner of the traditional MIPs, which could influence the adsorption efficiency, and the materials with large surface area and high porosity were used as support matrices for the preparation of MIPs through surface-imprinting polymerization. The surface-imprinted functionalized silica gel sorbent was prepared, and the surface-imprinted polymers exhibited with a great selective adsorption to patulin. The surface-imprinted sorbent had been resoundingly applied to online SPE coupled with HPLC to determine the trace levels of patulin in fruit-derived samples (Yang et al. 2016).

Currently, the majority of nonthermal methods were used to reduce the level of patulin in fruit juice. The microbiological quality of food could be improved through the high hydrostatic pressure (HHP). The main advantage of HHP treatment was the better nutritious quality of the yielding products than those processed with traditional methods, while the organoleptic properties of food were unaffected (San Martin et al. 2002). The levels of patulin in apple juices and concentrates could be reduced to 62 ppb by the high hydrostatic pressure treatment of 600 MPa for 300 s at 11 °C (Hao et al. 2016). The impact of pulses, single pulse of high hydrostatic pressure treatment were compared. The decrease of patulin level in fruit juice was up to 62.11% with the pressure treatment in combination with pulses and mild heat (Avsaroglu et al. 2015). The efficacy of pressure was related to the applied pressure and the patulin concentration in food. The HHP application for patulin elimination was more effective at high concentrations, while pulse-HHP application was more effective at low concentrations.

In addition, the most challenging questions for chemical methods could hamper its application in food industry, including chemical detoxification, ambiguous degradation mechanism, and hazardous concerns. The biological strategies for patulin elimination were less expensive but more effective. Furthermore, lots of microorganisms could be safely applied to food industries. A number of microorganisms have been studied to remove patulin from fruit juice, and so far the biological method mainly focuses on yeasts (Dong et al. 2015), *Alicyclobacillus*, lactic acid bacteria, and other bacteria. Yuan et al. found that the removal of patulin by *Alicyclobacillus* was strain-specific. The highest patulin reduction rate of 88.8% was achieved after 24 h incubation with initial patulin concentration of 100 μg/L and bacteria powder amount of 40 g/L (Yuan et al. 2014). The patulin-degrading activity of *Rhodosporidium paludigenum* was inducible by patulin. Patulin could be completely degraded by intracellular protein from lyophilized yeast cells in 1 h, and the transformation product was probably desoxypatulinic acid. The *Rhodosporidium paludigenum* or its purified enzymes could be used for the detoxification of patulin in fruit juice (Avsaroglu et al. 2015). The biodegradation of patulin by *Byssochlamys nivea* strain was also researched. The patulin degradation by *Byssochlamys nivea*

strain was studied under different conditions, indicating that the optimum temperature was 37 °C and pH had little effect on patulin degradation (Zhang et al. 2016).

10.2.3.3 New Methods of Removing Ochratoxin

The molecularly imprinted biocompatible magnetic nanoparticles for specific recognition of ochratoxin A was studied (Turan and Şahin 2016). In this research, functional monomer OEGMA, cross-linker 1,2-ethylene glycoldimethacrylate (EGDMA), template molecule OTA, initiator 3-bromopropyltrimethoxysilane (BPTS), and Cu (I) brand 2,2-bipyridyl (2,2-bpy) were chosen for the synthesis of the MIP layer on the MNP surface. MIP and MNPs showed very fast and large adsorption abilities, and it owned a high selectivity to OTA.

Physical, chemical, and biological strategies have been developed to decontaminate biotoxins in food commodities. Physical strategies, such as heat treatment, gamma-ray irradiation, and electron beam (EB) irradiation, are usually effective in degrading biotoxins from contaminated food commodities (Jalili et al. 2010; Zhang et al. 2007). OTA is very stable, and only 20% of OTA in wheat can be degraded by dry heat at 150 °C for 32 min or 100 °C for 160 min (Boudra et al. 1995). For evaluating the effects of dose of gamma ray ranging from 0 to 60 kGy, the response surface methodology (RSM) was applied, and the maximum reduction rate of 52% for OTA was found at 60 kGy (Jalili et al. 2010).

In addition, the degradation of OTA in aqueous solutions by electron beam (EB) irradiation was investigated (Peng et al. 2015). EB irradiation has a higher effective capacity of degrading OTA in water than that in acetonitrile or methanol–water. The degradation efficiency of OTA is enhanced in an irradiation dose-dependent manner and decreased when the substrate concentration was enhanced. The alkaline conditions (pH 9.99) and the addition of H_2O_2 (<0.1% v/v) significantly improved the degradation efficiency of OTA. OTA degradation process was in accord with the pseudo first-order kinetic model. In addition, the degradation products were preliminary analyzed by liquid chromatography–mass spectrometry.

10.3 Potential of LAB to Reduce Biotoxins

10.3.1 Introduction

The biotoxin decontamination by physical and chemical strategies has been reviewed in lots of papers (Peng et al. 2015; Turan and Şahin 2016), and there were many limitations, such as the change of flavor, the destruction of nutrition, and the influence of food functional properties, and it may have an existence of toxic residues (Méndez-Albores et al. 2007). Thus, other safer techniques of biotoxin decontamination need to be developed, and among them microorganism-based biological

method is appropriate strategy. Indeed, numerous researches have shown that some of the microorganisms own the ability of removing the toxins from the environment.

LAB has lots of potential applications as one of the biological antagonists. This kind of microorganisms has been extensively used in the fermented foods and the commensal microflora in the gut. The LAB has a long history of safe use in foods. They can generate some antagonistic compounds to inhibit the growth of pathogenic bacteria and restrain the undesirable spoilage microflora. Some compounds which own strong antifungal activities have been isolated from the culture of LAB, and the majority of these active constituents are low-molecular-weight compounds that consist of organic acids, hydrogen peroxide, reuterin, hydroxyl fatty acids, proteinaceous compounds, and phenolic compounds.

10.3.2 The Application of Lactic Acid Bacteria in Reducing Effects of Biotoxins

10.3.2.1 The Study of LAB on Reducing Effects of Aflatoxin

LAB are usually used in silage feed and fermented food, and they are known to not only control the fungal growth but also increase the shelf life of products (Broberg et al. 2007). Numerous LAB strains can inhibit the growth of aflatoxin-producing fungal strains, and the antifungal activities are influenced by several factors including the species, incubation period, temperature, culture medium, the production of antifungal compounds, and pH (Ahlberg et al. 2015). A *Lactobacillus casei* strain, isolated from a commercial silage culture, was able to restrain the growth of fungi or reduce the aflatoxin production in its living cultures and cell-free supernatants. The results demonstrated that the living cultures controlled the fungal growth and decreased the aflatoxin production, while the cell-free supernatants did not restrain the growth of fungi although it remarkably decreased the aflatoxin production (Gourama and Bullerman 1995). The inhibition zone of two *Lactobacillus fermentum* strains against aflatoxin-producing *Aspergillus* strains varied between 1 and 16 mm and between 5 and 12 mm, respectively (Onilude et al. 2005). *Lactobacillus fermentum* L23, another LAB strain with the probiotic specialities, was also tested against *Aspergillus flavus* of ten different strains to evaluate its influences on the growth inhibition and aflatoxin production reduction (Gerbaldo et al. 2012). The results showed that *Lactobacillus fermentum* L23 inhibited nine of the tested strains, and the inhibition rate of the fungal growth enhanced from 36% to 50% compared with the control. Correspondingly, aflatoxin production decreased between 73% and 99%. *Lactobacillus rhamnosus* L60 was effective against ten different *Aspergillus flavus* strains. The results showed that the LAB strain inhibited the fungal growth partially or totally. The rate of growth decreased from 77% to 96%, and the aflatoxin B1 production decreased between 96% and 99% (Gerbaldo et al. 2012).

A great many of published studies mainly focused on *Lactobacillus plantarum* strains inhibiting mycotoxin. It is reported that the culture and the cell-free supernatant of *Lactobacillus plantarum* NCDO 1752, isolated from commercial silage, inhibited mycelia growth and reduced aflatoxin production (Gourama and Bullerman 1995). Vanne et al. found the inhibition rate of *Lactobacillus plantarum* VTT E-78076 and VTT E-79098 against fifteen *Penicillium verrucosum* and *Aspergillus ochraceus* strains varied from 28% to 50% (Vanne et al. 2001). Another research studied the inhibition ability of the same two strains against four different *Fusarium* species and the inhibition rate varied from 33% to 53% for VTT E-78076 and 30% to 50% for VTT E-79098 (Laitila et al. 2002). This study also analyzed the inhibitory activities of *Lactobacillus plantarum* VTT E-78076 against *Fusarium* fungi in naturally infested steeped barley and malt, while the *Fusarium* growth was reduced by 20–50% in barley and by only 5–17% in final malts. *Lactobacillus plantarum* MW and YO isolated from indigenously fermented cereal gruels could inhibit the growth of four *Aspergillus flavus* strains and two *Aspergillus parasiticus* strains in different degrees (Onilude et al. 2005). When *Lactobacillus plantarum* C21-41, isolated from durum wheat semolina samples, was inoculated with *Aspergillus niger*, this fungus growth was inhibited by 42% (Valerio et al. 2009).

The antimicrobial compounds from *Lactobacillus plantarum* strains were widely investigated, and these compounds contain phenyllactic acid and its 4-hydroxy derivate (Valerio et al. 2004). Other identified antifungal metabolites from *Lactobacillus plantarum* strains were catechol and azelaic acid (Broberg et al. 2007). An antifungal substance 3,6-bis(2-methylpropyl)-2,5-piperazinedione identified from *Lactobacillus plantarum* AF1 completely inhibited *Aspergillus flavus* growth in soy beans for 2 days (Yang and Chang 2010). Furthermore, the inhibition of the fungus growth is better in the cell-free supernatant and a combination of organic acids. Ndagano et al. studied the antifungal impacts of the separate organic acids which were produced through LAB and found that inhibitory activity was gained by a synergy of these compounds (Ndagano et al. 2011). Individual lactic acid needed a concentration of 1220 mM to control the growth of fungi, while the production level of LAB is approximately 76 mM, and the inhibitory activity is also affected by pH. The experiment of synergic inhibitory activity was performed against three *Aspergillus* strains; the results showed that the growth inhibition level reached more than 75% with a combination of acetic acid, lactic acid, and phenyllactic acid at the concentrations of 33.3, 333, and 2 mM, respectively. The study of Laitila et al. also demonstrated that lactic acid alone inhibited the growth at best 20% but mostly no more than 10%, which indicated that lactic acid or low pH alone was not able to elucidate the inhibition activity of *Lactobacillus plantarum* (Laitila et al. 2002). Therefore, except for organic acids, other antifungal compounds which were produced by LAB were also able to make a contribution to the inhibition activity and synergistic effect.

10.3.2.2 The Study of LAB on Reducing Effects of Patulin

Attempts had been made to using new strategies to eliminate patulin in the contaminated food or degrade this toxin into less toxic substance. However, these strategies were expensive or unrealistic for some regulatory limitation. Another strategy was using microorganisms to adsorb patulin, since many LAB strains were food-grade microorganisms and had been used for several decades as natural biopreservatives in food. Patulin was considered as an antibiotic which was primarily produced by *P. expansum*. The growth of LAB strains could be inhibited at very high patulin concentrations (100 mg/mL). Recently, many researchers reported that LAB could restrain the germination of mold spores, retard the growth of mycelia, and decrease the biotoxin (such as aflatoxins, ochratoxin, and patulin) in food products (Topcu et al. 2010; Elsanhoty et al. 2014; Serrano-Niño et al. 2015; Piotrowska 2014).

There were some researches on the reduction of patulin in aqueous solution by LAB. The LAB strains with patulin adsorption activity were isolated from the fermented foods. The *Bifidobacterium bifidum* and *Lactobacillus rhamnosus* strains were shown to have certain patulin uptake activity, and the highest removal activity of patulin was accomplished at pH 4.0 and 37 °C. The removal percentage of patulin from aqueous solution was significantly increased with incubation times. The removal percentage of patulin ranged from 8.1% after 1 h to 19.6% after 24 h incubation for viable cell. The patulin removal was decreased with the increment of patulin, and the highest removal of patulin was observed at pH 3.0 and 4.0. In addition, the removal of patulin by LAB was influenced by temperature, and the maximum removal was observed at 37 °C for *Bifidobacterium bifidum*. However, the *Lactobacillus rhamnosus* strain had a maximum removal ability at 25 °C, and the patulin concentration obviously influenced the percentage of the toxin bound. The patulin adsorption rate decreased as the level of patulin increased (Hatab et al. 2012a).

In our studies, 48 LAB strains were used for measuring the removal capability of patulin. *Lactobacillus plantarum* LB-11, as the most efficient LAB, could remove more than 90% patulin within 24 h. As shown in Fig. 10.5, *Lactobacillus plantarum* LB-11 strain had good tolerance to patulin and was able to grow normally in MRS medium at a concentration of 1000 μg/L, 5000 μg/L, and 10000 μg/L.

Some researches focused on the patulin adsorption by the inactivated LAB for preventing bacterial contamination (Wang et al. 2015b). In addition, the key factors of patulin adsorption through inactive LAB strains were studied. The research showed that alkaline amino acids, thiol, and ester compounds were very critical for the patulin adsorption by LAB. Besides hydrophobic interaction, electrostatic interaction also takes part in the adsorption of patulin (Wang et al. 2015a). The patulin adsorption by the LAB was decreased with trypsin and pepsin pretreatment. It was demonstrated that aspartic acid was the main determinant for the trypsin narrow specificity, and the hydrophobic walls of the pocket created an appropriate condition for the long aliphatic and unbranched parts of the fundamental arginine and lysine side chains. Hence, the adsorption sites of patulin could include the alkaline amino acids.

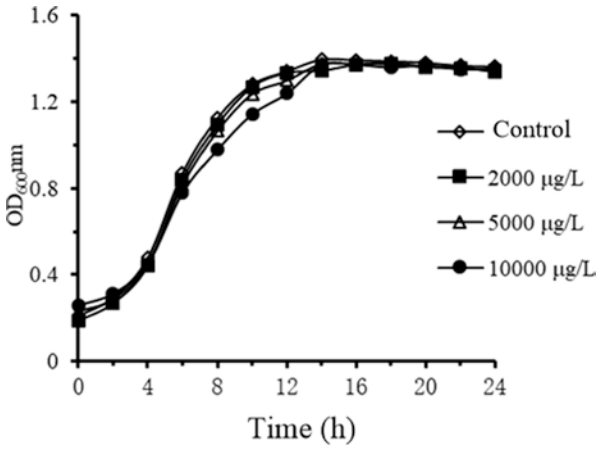

Fig. 10.5 Patulin tolerance capacity of *Lactobacillus plantarum* LB-11

The patulin elimination in the liquid medium using different LAB strains was also analyzed by HPLC or fluorescence detectors (Fuchs et al. 2008). The result showed that *Bifidobacterium animalis* exhibited the efficient patulin adsorption, and the patulin adsorption of *Bifidobacterium animalis* was affected by various parameters, such as the toxin concentration, the cell density, pH, and the survival rate of the LAB strain. The maximum removal of patulin by *Bifidobacterium animalis* was observed at pH 5.0, while the LAB binding ability to biotoxins was alike at a broad range (El-Nezami et al. 2004).

10.3.2.3 The Study of LAB on Reducing Effects of Ochratoxin

Currently, most studies dealing with the biopreservation activity of LAB strains were focused on their antibacterial effects. There is an increasing interest toward LAB strain adsorption/removal of OTA (Piotrowska 2014; Piotrowska and Zakowska 2005; Schnürer and Magnusson 2005; Shetty and Jespersen 2006; Turbic et al. 2002; Dalié et al. 2010; Belkacem-Hanfi et al. 2014). However, it was noted that mycotoxins exhibited antibiotic properties and inhibit the growth of microorganisms. Therefore, bacterial species not susceptible to OTA in the environment should be selected in the decontamination researches. LABs are usually not very susceptible to OTA, but there are some differences between the species and strains (Piotrowska and Zakowska 2005). Twenty-nine strains of the *Lactobacillus* and *Lactococcus* genera were used to eliminate OTA. The capacity of reducing the amount of OTA is common among LAB strains, but it often varied depending on the species and the strains. The maximal decrease, more than 50% of the OTA amount, was found in the strains *Lactobacillus acidophilus* CH-5, *L. rhamnosus* GG, *L. plantarum* BS, *L. brevis*, and *L. sanfranciscensis*. A sharp decrease of OTA was discovered during the first 15 h in the culture growth. The OTA elimination process is partly reversible, and part of OTA is released back into the medium after 40 h of

incubation. And a part of the OTA becomes bound by the LAB strains. In addition, the amount of OTA eliminated in the bacterial growth was found to vary between 8% and 28%, while no degradation product was detected, indicating that OTA elimination using wine LAB was a binding procedure (Del Prete et al. 2007). In addition, the differences of LAB strains with respect to aflatoxin binding suggest that the binding ability has an extremely high strain specificity (Turbic et al. 2002).

10.3.2.4 The Study of LAB on Reducing Effects of Microcystin-LR

Cyanobacteria toxin-induced worldwide illness and deaths occurred in humans and animals (Dawson 1998; Azevedo et al. 2002). MC-LR, a most common and toxic cyanobacteria toxins, was considered to be able to cause liver damage, reproductive toxicity, kidney injuries, gastrointestinal apoptosis, and immunosuppression (Wu et al. 2014; Botha et al. 2004; Guzman and Solter 2002; Lei and Song 2005; Shen et al. 2003). To date, the strategy of decreasing MC-LR exposure prevailingly contains removal, degradation, and reduction on the intake of MC-LR. It was reported that MC-LR might be degraded by LAB, and the cell-envelope proteinases involved in the degradation of MC-LR had been identified in *Lactobacillus* and *Bifidobacterium* strains (Nybom et al. 2012). The elimination of MCs using LAB in vitro was a presumptive physical adsorption (Fuchs et al. 2008; Piotrowska 2014; Hatab et al. 2012b).

Large numbers of LAB strains have been reported to own the abilities of free radical scavenging, metal ion chelating, and prooxidative enzyme inhibition (Lin and Yen 1999; Achuthan et al. 2012). Among LAB strains, several selected *Lactobacillus* strains played a vital role in alleviating oxidative stress in vivo (Zhang et al. 2010a, b). Moreover, recent researches indicated that some *Lactobacillus* and *Bifidobacterium*, such as *Lactobacillus rhamnosus*, *Bifidobacterium breve*, and *L. plantarum*, could eliminate MC-LR or decrease its toxicity in vitro (Halttunen et al. 2008; Nybom et al. 2008; Surono et al. 2008).

Our previous research indicated that single LAB strain didn't have a significant effect on MC-LR-exposed mice, although the strain had been demonstrated to have a nice adsorption ability of MC-LR and strong antioxidative capacity in vitro. Then, we selected 20 LAB strains from the list of eatable bacteria released by National Health and Family Planning Commission of the People's Republic of China and evaluated the cocktail of LAB against MC-LR-induced oxidative stress and hepatotoxicity in mice. Our results showed the cocktail which contained *Lactobacillus johnsonii* ATCC 33200, *L. rhamnosus* GG, *Bifidobacterium adolescentis* 1.1290, and *B. bifidum* CCFM 16 was able to obviously restrain the increased levels of serum ALT and AST (Fig. 10.6). The changes of hepatic malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) activities in this cocktail were also improved (Fig. 10.7). Moreover, the cocktail had a high free radical scavenging ability and alleviated MC-LR-induced hepatic damage. Thus, a cocktail of LAB could be a promising dietary strategy to restrain the cyanobacteria contaminant toxicity (Zhao et al. 2017).

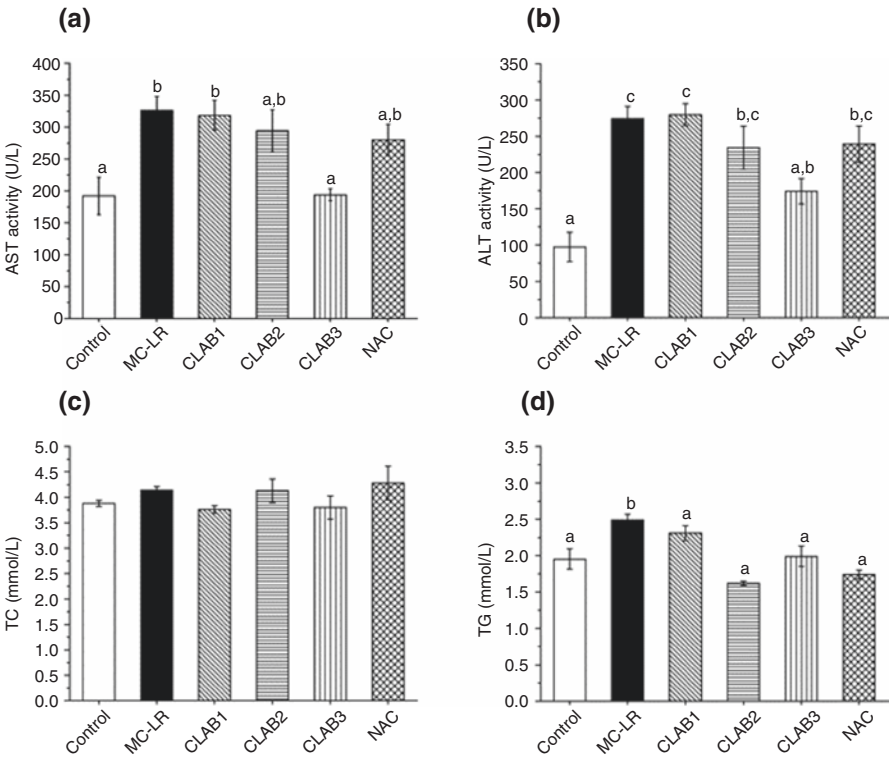

Fig. 10.6 Effects of a cocktail of LAB on the serum enzyme and biochemical indicates: (**a**) AST activity, (**b**) ALT activity, (**c**) TC contents, and (**d**) TG contents (Zhao et al. 2017)

To study the effects of LAB on MC-LR-induced toxicity, the direct contact between MC-LR and the mixed LAB was avoided through administration of MC-LR via intraperitoneal injection rather than intragastric route. The liver is the target organ by MC-LR as the uptake of this toxin needs to be by the bile acid carrier system in hepatic cells (Fawell et al. 1999). MC-LR restrains the protein serine/threonine phosphatases 1 and 2 A by its methyl-dehydroalanine-binding Cys^{273} of the phosphatases, resulting in an excess of protein phosphorylation and subsequent hepatocyte damage (Toivola et al. 1994; Nishiwakimatsushima et al. 1992; Mackintosh et al. 1995). Our study indicated that liver damage was given rise to a sublethal dose of MC-LR and increased the levels of serum ALT and AST, but it was restrained when oral administration of LAB cocktail was performed. It was also reported that LAB with high antioxidative activity can increase the glutathione peroxidase level that was important for the detoxification of MC-LR (Wang et al. 2016; Gehringer et al. 2004). In addition, research has indicated that oxidative stress played a key role in the pathogenic mechanism of MC-LR-exposed toxicity (Ding and Ong 2003). Reactive oxygen species mainly come from the transport chain in mitochondria and enzymes in oxidative phosphorylation. The oxidative stress can

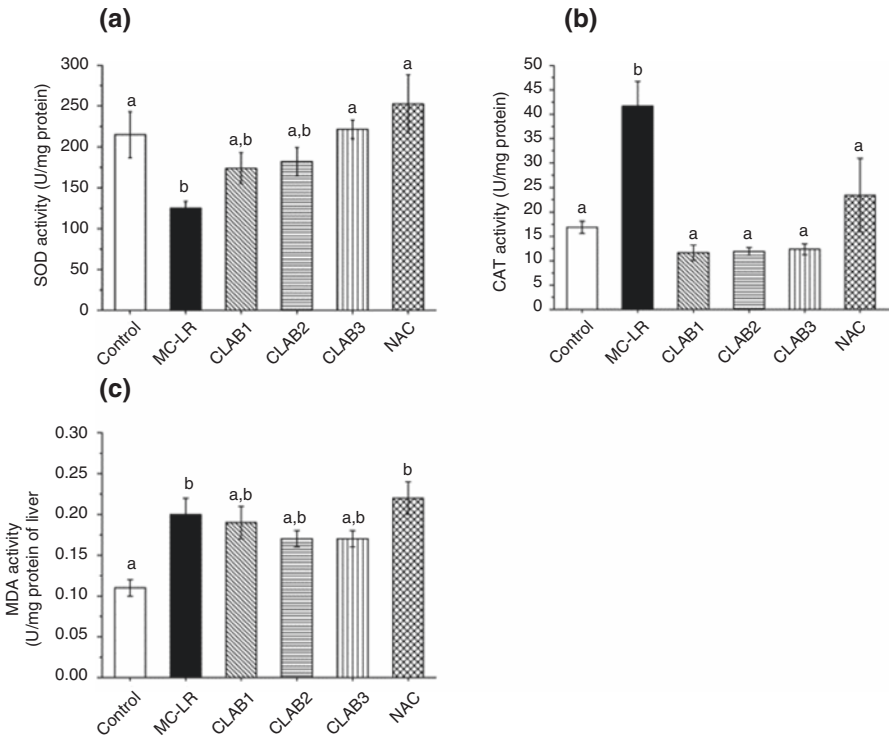

Fig. 10.7 Effects of a cocktail of LAB on the antioxidant enzymes in livers of MC-LR-exposed mice: (**a**) SOD activity, (**b**) CAT activity, and (**c**) MDA contents (Zhao et al. 2017)

induce serious damage of cell structures through promoting the destruction of lipids, proteins, and DNA when the balance between reactive oxygen species and antioxidative system is broken (Zhao et al. 2017). Indeed, some *Lactobacillus* and *Bifidobacterium* have been reported to have the antioxidative capacities of decreasing lipid peroxidation, enhancing antioxidative enzyme, and improving lipid metabolism (Zhang et al. 2010a, b).

10.3.3 The Mechanism of Biotoxins Adsorption Using Lactic Acid Bacteria

10.3.3.1 The Mechanism of Aflatoxins Adsorption Using LAB

Some lactic acid bacteria (LAB) strains can bind mycotoxins, and most of the binding researches were performed at optimal experimental conditions with some strains of different LAB species (Ahlberg et al. 2015; Blagojev et al. 2012; Gratz et al. 2004). The adsorption process of aflatoxin by LAB is forming bacteria–aflatoxin

complex. With the decline of LAB adsorption ability, the LAB is easily excreted together with aflatoxin, thereby reducing the harmful effects of toxins on human body (Dalié et al. 2010).

Previous study showed *Lactobacillus rhamnosus* GG and LC-705 had a remarkable effect on removing aflatoxin B1 and it was able to trap more than 80% of the toxin at 20 μg/ml solution (El-Nezami et al. 1998). El-Nezami et al. also studied the ability of these two strains of *L.* GG and LC-705 and a *Propionibacterium* spp. to remove aflatoxin B1 from a chicken intestinal luminal liquid medium and found that the average removal efficiency of aflatoxin was 54% within 1 min (El-Nezami et al. 2000). Further investigations on the toxicity and transport of aflatoxin B1 binding by *Lactobacillus strain* GG were performed using Caco-2 cells, and the results suggested that *Lactobacillus strain* GG decreased the uptake of aflatoxin B1 and protected itself against the damage of membrane and DNA (Gratz et al. 2007). The detoxifying prospects with five different LAB cultures were studied for aflatoxin B1 detoxification, and the results demonstrated that reduction of aflatoxin B1 concentration was up to 45% (Oluwafemi et al. 2010). The research of potential effect on *Lactobacillus casei Shirota* protecting against aflatoxin B1 using a murine model suggested that this strain was able to bind aflatoxins at intestinal level and prevent the toxin absorption (Hernandez-Mendoza et al. 2010). Hathout et al. also observed that *Lactobacillus casei* and *Lactobacillus reuteri* can provide protection against aflatoxin B1-induced oxidative stress, indicating that the dietary treatment with the strains after aflatoxin-contaminated diet successfully protected against the injuries of the liver and kidney in experimental animals due to their binding and antioxidant properties (Hathout et al. 2011).

As LAB have diverse properties and various cell wall compositions, the binding of aflatoxin through LAB showed exceeding strain-specific (Ahlberg et al. 2015). In LAB, the cell wall consists of various components (e.g., polysaccharides, peptidoglycan, S-layer protein) which have functions including adhesion and macromolecular binding (Delcour et al. 1999). Haskard et al. found that aflatoxin B1 predominantly bound with the carbohydrate components in the cell wall and some proteins were also involved in the binding (Haskard et al. 2000). This research also demonstrated that the binding still occurred although there were some pretreatments including heat and acid treatments, suggesting multiple components in cells were involved in the binding. Another study also supported the conclusion that the binding appearance was not given rise to one factor but various systems of cell wall components (Hernandez-Mendoza et al. 2009a). Moreover, it was reported that aflatoxin B1 was bound to bacteria by weak non-covalent interactions and the multiple components in the cell wall of LAB were involved in the binding (Peltonen et al. 2001; Turbic et al. 2002). Autoclaving resulted in denaturation of bacterial proteins and enzymes, but it didn't lead to the release of the most strongly bound aflatoxin B1, indicating that the toxin was not restrained to loosely attached the components of bacterial cell wall (Haskard et al. 2001).

In all probability, aflatoxin binding is a surface phenomenon, with a remarkable involvement of carbohydrates, peptidoglycan, and proteins. However, cellular lipids were not seemed to be involved in the binding procedure (Haskard et al. 2000).

Furthermore, Pierides et al. found that the heat-killed LAB cells have a better binding effect with aflatoxin M1 than that of the viable cells (Pierides et al. 2000). The bacteria cell wall compounds have an obvious effect on the bond formation, and the nonviable strains owned a better binding activity than that of live bacteria. This may be due to the fact that the bacteria metabolism reduces the binding effect between bacteria cell and toxins (Ahlberg et al. 2015). Thus, the binding effect of aflatoxins by LAB is probably multiple individual factors, and thus it needs to be further elucidated on the binding mechanisms.

10.3.3.2 The Mechanism of Patulin Adsorption Using LAB

For investigating the mechanisms of the mycotoxins removal through LAB, the impacts of viable and heat-inactivated bacteria were compared in a lot of research (Haskard et al. 2001; El-Nezami et al. 2002). However, the adsorption interaction mechanism between the LAB cells and patulin remained unclarified. In recent studies, various physical, chemical, and enzymatic treatments were used to confirm the site of potential adsorption to biotoxins on bacterial cells. The results indicated the adsorption procedure was on account of the non-covalent interactions between the toxins and the cell wall carbohydrates and protein moieties (Dalié et al. 2010; Topcu et al. 2010; Guo et al. 2012). Moreover, the integrity of the bacterial cellular structure was critical for the biotoxin adsorption (Guo et al. 2012; Pizzolitto et al. 2012). Therefore, the surface characteristics of integrated bacterial cells were the very key factors, and it needed to be further studied.

Improvement in the adsorption capacity of LAB strains to patulin was frequently bound up with an increase in specific surface area. The patulin adsorption of LAB varied between the different *Lactobacillus*, and this might be the differences of the structures in their cell wall and cell membrane. The patulin adsorption was influenced by the LAB cell surfaces. The changes of cell surface were detected by FTIR spectra, and the patulin-exposed LAB cells didn't entirely lose the original structure. The obvious changes were mainly on the C-O, O-H, and N-H groups. The cell wall polysaccharides and proteins were critical components in patulin adsorption by LAB strains through the EDS and FTIR analysis (Wang et al. 2015b), and the results were consistent with other reports (Guo et al. 2012; Hatab et al. 2012b). In addition, the influences of zeta potential and surface hydrophobicity of LAB cells were verified on the patulin adsorption. The results showed that surface hydrophobicity was more beneficial than the surface charge to the adsorption of patulin (Wang et al. 2015b).

A variety of physical, chemical, and enzymatic treatments have been used to verify the characteristic of the interactions between biotoxins and LAB cells. It was well known that urea was an anti-hydrophobic agent and the treatment of LAB strains with urea showed a significant effect on the biotoxin adsorption. The hydrophobic interactions played a key role in the biotoxin adsorption (Guo et al. 2012; El-Nezami et al. 2004). The lipoteichoic acids and envelope proteins were critical to the hydrophobicity of cell surface, and this was beneficial to the patulin adsorption by LAB strains (Wang et al. 2015b).

The patulin adsorption by LAB cells varied among the investigated bacteria strains, and this may be due to the structural differences of the cell wall and cell envelope. The mechanism of patulin adsorption through inactive cells had been studied, and the results showed that the carbohydrate and protein moieties and hydrophobic interactions were vital for the patulin adsorption (Peltonen et al. 2001). There were differences between bifidobacteria and lactobacilli in the phospholipid composition, especially in the polyglycerol phospholipids and the aminoacyl-phosphatidylglycerol. The maximum patulin adsorption of *Bifidobacterium bifidum* and *Lactobacillus rhamnosus* strains arrived at 52.9% and 51.1% at 1 mg/L of patulin, respectively (Hatab et al. 2012a).

The mechanism of adsorption patulin through LAB was studied by SEM-EDS, TEM, BET, and FTIR techniques, and the results showed that the major functional groups associated with adsorbing patulin were C-O, OH, and NH groups. The polysaccharides and protein were the important functional components for patulin adsorption. The patulin adsorption capacity of LAB was closely related to the physical and chemical speciality of cell surface, containing cell wall volume, specific surface area, the ratio of nitrogen/carbon, hydrophobicity, and functional groups (Wang et al. 2015b). On account of these results, it was speculated that the elimination of patulin was owed to the non-covalent binding between the toxins and the carbohydrate moieties of cell walls.

In our studies, main mechanism of patulin-removing capability by *L. plantarum* LB-11 was also explored. The patulin removal efficiency of viable cells was compared with inactivated cells, as well as different cell components including cell pellets, cell walls, and cell extracts. The patulin removal efficiency of inactivated cells, cell walls, and cell extracts was significantly lower than intact living cell pellets, indicating that the patulin-removing capability of *L. plantarum* LB-11 mainly depended on the intact living cells (Fig. 10.8).

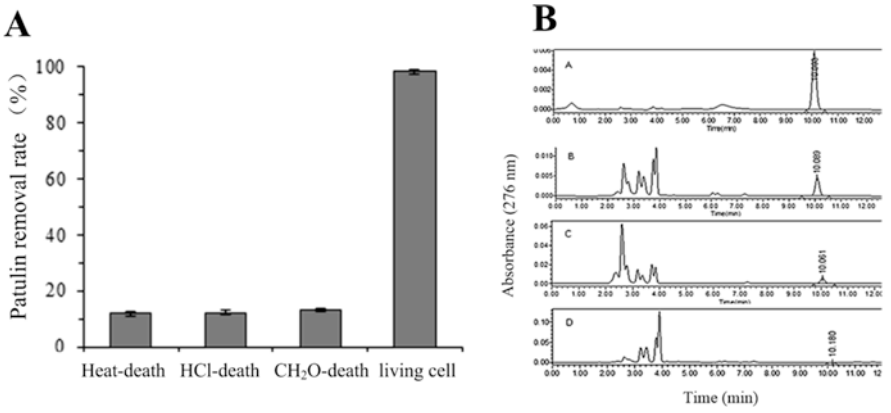

Fig. 10.8 Influence of cell activity of patulin-removing capacity (**a**) and the HPLC chromatograms of patulin removal ability of both cell wall and intracellular extract (**b**)

10.3.3.3 The Mechanism of Ochratoxin Adsorption Using LAB

The adsorption of OTA by LAB strains is a prospective strategy for the food decontamination. Some studies have been performed to explain the mechanism of orchatoxin adsorption by LAB strains (Piotrowska 2014). Three LAB species, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus sanfranciscensis* had been used to adsorb OTA. It has been reported that OTA was eliminated from media when the active cells exist, indicating that the toxin was adsorbed to the cells. Heat-inactivated cells revealed decontamination specialities, which were several times higher than that of live biomass at the same concentration. Furthermore, it has been found that OTA was able to be released on the effect of washing with water or acid, suggesting the binding between the toxin and biomass was not very strong. The binding of toxin-to-cell might be of physicochemical speciality. The adsorption between the OTA and the cell wall surface structures is due to the hydrophobic nature of cell wall, the electron donor–acceptor, and Lewis acid–base interactions. Nevertheless, the differences between the LAB cell wall components were not studied.

10.3.4 The Degradation of Biotoxins by Lactic Acid Bacteria

The degradation of patulin had been demonstrated in fermented cider using yeast (Moss and Long 2002) and *Gluconobacter oxydans* (Ricelli et al. 2007). Recently, the patulin adsorption had been shown with *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* strains (Hatab et al. 2012a), and *L. acidophilus* (Fuchs et al. 2008). The patulin degradation of LAB strains had gradually been characterized, and the patulin degradation by *L. plantarum* seemingly followed the well-demonstrated pathway to ascladiol (Hawar et al. 2013).

The microorganisms could be applied for detoxicating patulin to potentially weaker toxic compounds (Castoria et al. 2011). So far two main products of biological degradation of patulin were ascladiol and desoxypatulinic acid (DPA) (Fig. 10.9). Both ascladiol and DPA have a mass of 156 Da and have a maximum UV absorption at 268 nm because they own the same molecular formula with $C_7H_8O_4$ (McCormick 2013). Desoxypatulinic acid was short of the functions of hemiacetal and lactone and cannot react with thiol groups. Therefore, the toxicity of desoxypatulinic acid

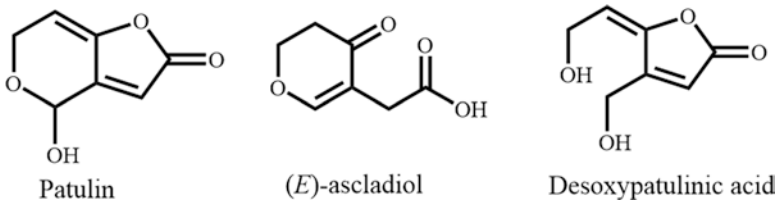

Fig. 10.9 Patulin, E-ascladiol, and desoxypatulinic acid

was much weaker than that of patulin in the human lymphocytes assay (Castoria et al. 2011).

The biotransformation product of patulin by *Lactobacillus plantarum* was likely ascladiol (Hawar et al. 2013). The patulin degradation by *L. plantarum* was pH dependent, and its degradation activity was weak at low pH (pH = 3). For further investigation of the patulin biotransformation pathway by LAB strain, the metabolite analysis of patulin by HPLC was performed. The main patulin metabolite by *L. plantarum* was ascladiol. The patulin degradation of *L. plantarum* appeared to occur not only in the cell-free supernatants but also in homogenized cells, whereas the patulin degradation activity was decreased after heating at 60 °C and removed out by autoclaving (Hawar et al. 2013). After a long-term incubation of *L. plantarum* strain with patulin at 4 °C for 4 weeks, the main metabolite of patulin degradation was not the ascladiol isomers, although both *E*- and *Z*-ascladiol products were present. The compound was identified as 5-(2-hydroxyethyl)-4-(hydroxymethyl) furan-2(5H), which lacked the double bond which was responsible for the geometric isomerism in ascladiol. This novel metabolite was named hydroascladiol. In addition, this novel metabolite did not arise when LAB was shortly incubated with patulin, indicating that the ascladiol conversion to hydroascladiol was not mediated by the LAB strains and it could be a chemical reaction (Hawar et al. 2013). In our research, after detoxification of patulin by living *L. plantarum* LB-11, a new peak appeared in the HPLC chromatogram. By the use of LC-MS, the peak was presumably corresponding to E-ascladiol based on the retention time and fragments, indicating that the mechanism of patulin removal was mainly biodegradation (Fig. 10.10).

Decontamination or detoxification process of OAT has been considered, and indeed some physical, chemical, and biological approaches have been proposed for reducing OTA in musts and wine (Fuchs et al. 2008). Biodegradation is regarded as one of the most promising strategies to control mycotoxins. Numerous microorganisms are able to degrade and detoxify OTA (Ferenczi et al. 2014; Fuchs et al. 2008). Especially, several microorganisms have a very strong degradation ability, and it was considered as a promising method used to reduce the contamination of OTA in food or feed.

The microbiological degradation process of OTA needed many enzymes to take part in (Piotrowska and Zakowska 2005; Wu et al. 2011). Nevertheless, very little information of these enzymes was understood, and very few of them have been purified or characterized. The first reported protease which was identified to be capable of hydrolyzing OTA was carboxypeptidase A (CPA) (EC 3.4.17.1), which was from bovine pancreas, and it can release L-β-phenylalanine and ochratoxin α (OTα) (Wu et al. 2011; Ferenczi et al. 2014). OTα is thought of as a nontoxic compound, and it has an elimination half-life of tenfold shorter than OTA. There has been some studies reported that the degradation of OTA by many different microorganisms, such as *Trichosporon mycotoxinivorans* (Molnar et al. 2004; Schatzmayr et al. 2003), *Brevibacterium* spp. (Rodriguez et al. 2011), *Aspergillus* spp. (Stander et al. 2000), and *Cupriavidus basilensis* (Ferenczi et al. 2014).

The LAB strains were focused on the bacteria which have the capacity of eliminating OTA because of their safety and their better prebiotic functions on human health. The OTA concentration of milk was reduced when fermenting with yogurt

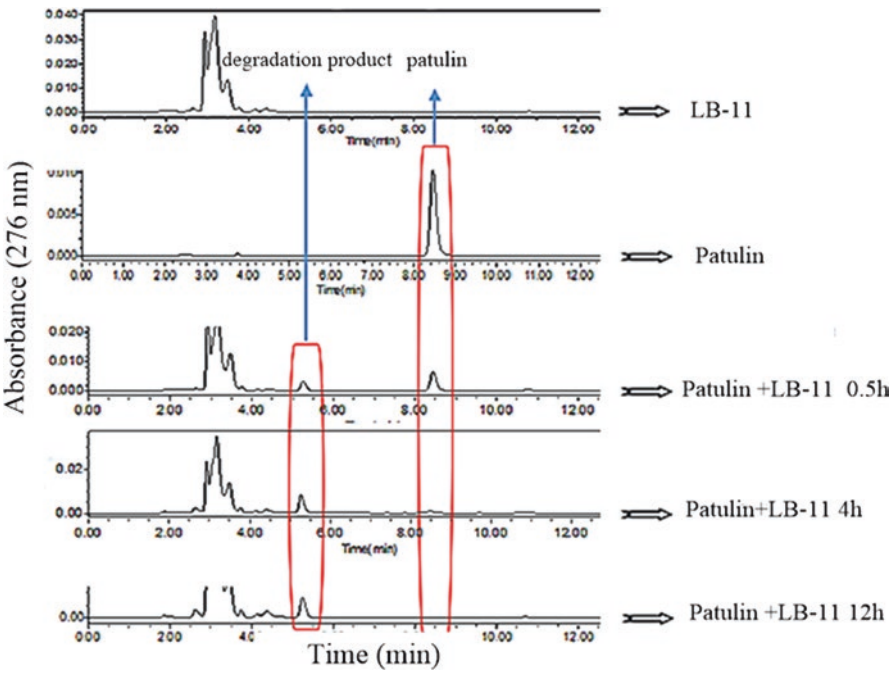

Fig. 10.10 The degradation chromatogram of patulin in different periods

bacteria and bifidobacteria. Indeed, many researches showed that the residues of OTA were not detected in those samples which were fermented by yogurt bacteria (*S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*). In addition, the obvious degradation ability of LAB was demonstrated in other articles (Böhm et al. 2000; Fuchs et al. 2008). Furthermore, the removal of OTA was able to be partially reversible in the LAB (Piotrowska and Zakowska 2005). Nevertheless, the degradation products of OTA were not identified in this research. It is noteworthy that the elimination of OTA using LAB in wine was not by degradation but through cell binding (Del Prete et al. 2007).

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