Chapter 11
Safety Evaluation of Lactic Acid Bacteria

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Fermented food containing active lactic acid bacteria (LAB) has been consumed worldwide for nearly a thousand years. The initial reason for adding lactic acid bacteria to the food system is to reduce the overall pH as its characteristics of producing lactic acid in the natural fermentation process, thereby inhibiting the pathogenic microorganisms and food spoilage, as well as prolonging the food shelf life. With the development of functional food, LAB with different functional characteristics and physiological activities have been used in food fermentation, which not only prolongs the shelf life of food but also improves the functional quality of food. The main purpose of developing fermented food with LAB is to improve the health of consumers, so the concept of "probiotics" is introduced into fermented food, and safety evaluation is indispensable in such functional products.

Lactobacillus and *Bifidobacterium* are the most common LAB used in food at present, which do not have any pathogenic characteristics. In general, they are seldom involved in common infections (except for *Enterococcus*). However, some rare cases of infections, mainly preclinical assessments of new or mixed strains, have been reported. Infections usually occur in people with serious underlying diseases, such as immunocompromised people or patients with hepatitis (Besselink et al. 2008a). As a result, such infections are extremely rare with little data to support risk factors for the general population. Many LAB strains, such as *Lactobacillus* and *Bifidobacterium*, have a good safety record in daily life. These strains are also important components of gut microbiota in healthy people and play an important role in many metabolic processes. In view of the particularity of LAB in functional foods and their important role in human health, the International Dairy Foods Association (IDFA) has formulated a document on the safety assessment of microorganisms in foods. The safety of LAB commonly used in food, including *Lactobacillus* and *Bifidobacterium*, has also

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been stipulated in the Qualified Presumption of Safety (QPS) document established by the European Food Safety Agency (EFSA). Most of LAB keep a safety record so far, and even many products still maintain good monitoring data after they are on the market (Salminen et al. 2002).

In view of the importance of this issue, the safety of LAB, especially for the new and mixed LAB strains, without safety assessment and history of safe use, must be strictly and systematically evaluated. Strict prevention of food safety problems caused by microorganisms is important for the development of safe and healthy functional probiotic food containing novel active LAB, and it is also a challenge in current and future food development.

11.1 Development and Present Situation of Lactic Acid Bacteria Safety Evaluation

11.1.1 The History of Safety Evaluation of Lactic Acid Bacteria

11.1.1.1 Potential Pathogenicity and Toxicity of Lactic Acid Bacteria

Translocation and Bacteremia of Lactic Acid Bacteria

The migration ability of LAB is related to its pathogenicity, and the direct method to measure the migration translocation ability of LAB is to give probiotics orally and count the number of bacteria invading various organs and tissues, including the blood, liver, spleen, kidneys, and mesenteric lymph nodes. These studies have demonstrated the safety of probiotics. Kabeir et al. confirmed that *B. pseudocatenulatum* did not migrate into the liver and blood of mice after oral feeding strains (Kabeir et al. 2008). Moreover, all organs and tissues, including the blood, liver, kidneys, spleen, and mesenteric lymph nodes, were analyzed after 7 days of administration with *B. longum* BB536; the results showed that no bacteria (including *Bifidobacterium*) was found (Abe et al. 2010). For *Lactobacillus*, Paturi et al. pointed out that *Lactobacillus acidophilus* and *Lactobacillus paracasei* did not translocate to the spleen, liver, and blood of mice (Paturi et al. 2008). However, early trials were conducted in disease-free normal mice, a model for healthy people, and most probiotic-induced sepsis or bacteremia occurred in patients with disease, not in healthy people. Therefore, using immunodeficient animals as susceptible populations (including infants or immunodeficiency patients), such as models of leukemia, premature infants, severe AIDS, and postoperative patients, are more appropriate for detecting translocation and sepsis.

Subsequently, several studies established mouse model of immunodeficiency for observing bacterial translocation and subsequent septicemia (Kawahara et al. 2015; Matsumoto et al. 2008). Cyclophosphamide is an immunosuppressive agent which is used in cancer patients sometimes and can induce immune damage in mice. The

results indicated that these mice have low white blood cell counts and become prone to intestinal cell migration. In this model, *Pseudomonas aeruginosa* was given, and these bacteria were observed in the blood and liver of mice, and all mice died within a few weeks. *Bifidobacterium longum* BB536 was administered orally before and after administration of *P. aeruginosa* in the immunocompromised mouse. No *B. longum* BB536 was detected in the liver and blood of mice, and the mortality caused by *P. aeruginosa* was inhibited after administration of *B. longum* BB536. These findings suggest that probiotic testing is relatively safe even in patients with low immune function and bacterial migration. Therefore, immunodeficient animals can simulate the status of immunocompromised people such as premature infants, the elderly, and critically ill patients. It is important to study whether probiotics can be used in healthy patients.

All probiotics may be susceptible to bacterial migration in severely damaged bowel walls, such as severe gastrointestinal injury, major diseases, and extensive use of antibiotics. However, bacterial translocation is significantly distinct from septicemia or endocarditis. If translocated bacteria do not cause sepsis, endocarditis, infection, or any other disease, it will not threaten the health and can be removed from the body (Yazawa et al. 2000). Conversely, if translocated probiotic can cause sepsis or infection, they should not be used (even if their translocation ability is low).

Previous studies have reported that when the germfree mice were given oral administration of pathogenic *Escherichia coli* O-111, most mice died within a few weeks as *Escherichia coli* O-111 migration and the production of enterotoxin in the organs (Turroni et al. 2014; Yamazaki et al. 1985). However, when the germfree mice were treated with *Bifidobacterium longum* BB536, the strain was observed in organs such as the liver, kidney, and mesenteric lymph nodes in the first 4 weeks, but no mice died, but after 4–8 weeks of production of specific IgG and IgA, the strain was not detected in all organs (Turroni et al. 2014; Yamazaki et al. 1985). The results indicated that *B. longum* BB536 strain did not have infection side effects even though it migrated and it is still safe for patients at risk of bacterial migration (Yamasaki et al. 2012). After oral gavage *B. longum* BB536 to germfree mice, *B. longum* BB536 was found to be transferred to the liver, mesenteric lymph nodes, and kidneys by organ counting. It was detected in every organ during the first 2 weeks, but was not detected after 4 weeks; no disease or death was found in all mice. In addition, the strain has been used in immunocompromised patients or in conjunction with anticancer drugs, including cyclophosphamide, to confirm the efficacy of probiotics against monilial infection. Monilia proliferation in patients resulting from taking antileukemic drugs can be reduced by *Bifidobacterium* intervention, and probiotics do not have any side effect.

Lactic Acid Bacteria and the Production of Harmful Metabolites

Biogenic amines (BAs) are a group of small nitrogen compounds with biological activity formed by the decarboxylation of amino acids or the amination of aldehydes and ketones (Maijala et al. 1995). It is ubiquitous in fermented foods, such as

fermented meat products, fermented seafood, fermented dairy products, and fermented vegetables (Shukla et al. 2015; Cvetkovic et al. 2015; An et al. 2015; Piersanti et al. 2014; De Mey et al. 2014; Kuley et al. 2011; Bunkova et al. 2010). The BA types and contents in different products or different samples of congeneric products are different, and they are influenced by environmental factors and fermentation process (Cvetkovic et al. 2015; Piersanti et al. 2014; Li et al. 2014). BA in food is mainly from the decarboxylation of free amino acids by amino acid decarboxylase generated by microorganisms.

BAs are bioactive organic alkaloids with important physiological functions, such as brain activity, gastric acid secretion, immunity, cell growth, and differentiation (Jagu et al. 2015). BAs are maintained at normal concentrations in tissues and cells through constant biosynthesis and metabolic decomposition (Linsalata and Russo 2008). However, excessive intake of BAs can break this balance and cause cardiovascular and neurological damage, leading to elevated blood pressure, rapid heartbeat, increased blood sugar levels, excessive adrenaline secretion, and headaches, and sometimes mainly gastrointestinal disorders, such as vomiting, diarrhea, and abdominal spasm (Kovacova-Hanuskova et al. 2015). It is also pointed out that the content of BAs synthesized by the organism on its own can meet the basic physiological function requirements without external supplements as long as the amino acid intake is adequate (Kalac 2014). Because the toxicity of BAs varies, and the detoxification ability of different populations against BAs is also very different, so the requirements of BAs level in food vary from country to country.

It is reported that the probiotics with the ability of BA production in foods include *Enterococcus* and *Lactococcus*, tyramine-producing bacteria isolated from cheese (Ladero et al. 2010). Lorencov et al. isolated 81 LAB from dairy products and beer to test their ability to produce BAs (Lorencová et al. 2012). The results showed that 50 strains had decarboxylase activity; 70% of the strains isolated from beer had an excellent ability to produce tyramine. Most of the LAB had decarboxylase activity; these LAB can increase the content of BAs in food, thereby threatening food safety and consumer health. It is noteworthy that some probiotics, such as *Bifidobacterium* and *Lactobacillus rhamnosus*, can also increase the levels of BAs in foods. Yüceer et al. also found that 68% of enterococci isolated from Turkish sausages can produce tyramine, which may have an adverse impact on consumer health (Yüceer and Banu 2015).

The Mucin Degradation Activity of Lactic Acid Bacteria

Increased mucin degrading activity of bacteria leads to more permeability of intestinal wall and further bacterial translocation (Gork et al. 1999). On the one hand, some researchers have reported that probiotics have or do not have mucin-degrading activity. They have demonstrated that *L. rhamnosus*, *L. acidophilus*, and *Bifidobacterium lactis* do not decompose mucin in vitro. Abe et al. (2010) also studied the mucin degradation activity of different strains of *Bifidobacterium* (including *B. longum*, *B. breve*, and *B. longum* subsp. *infantis*). It was initially observed that

Bifidobacterium grew slower in the medium with mucin as the sole carbon source than in the glucose medium. Subsequently, they used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stool samples as a positive control; the results confirmed that the *Bifidobacterium* did not decompose mucin. On the other hand, although most of *B. longum* and all tested *B. pseudocatenulatum* do not have these properties, some bifidobacteria, such as *B. bifidum*, *B. breve*, and *B. longum*, have mucin-degrading activity and contain gene encoding for mucin-degrading enzyme (Egan et al. 2014; Ruas-Madiedo et al. 2008). Since mucin plays an important role in preventing bacteria from invading the intestine, it is important to determine whether probiotics used in food have mucin degradation activity, i.e., the ability to damage the mucin layer. These findings suggest that mucin decomposition activity should be investigated at the strain level.

Vesterlund et al. studied the extracellular matrix proteins, mucus, blood cell adhesion properties, as well as the avoidance of the ability of inducing respiratory infection and the resistance characteristics of human serum in polymorphonuclear leukocyte (PMN), of 44 strains isolated from feces, 5 strains isolated from blood, and 15 strains isolated from probiotic products (including 3 strains from dairy products) (Vesterlund et al. 2007). Among the tested strains, the strains adhering to collagen, fibrinogen, and mucus were specific, while the strains isolated from feces, blood, and probiotics showed no significant statistical difference. However, the probability of respiratory infection induced by strains from probiotics in PMN was lower than by those from blood ($P < 0.05$). In addition, there was a positive correlation between adhesion collagen and respiratory infection in strains from feces ($P < 0.05$). In the determination of serum resistance, probiotics showed a tendency to be less sensitive to human serum-mediated death than strains from feces ($P = 0.07$). Compared with fecal or probiotic strains, no detectable virulence factors were found in blood strains, suggesting that these factors do not pose a risk when considering the safety of probiotics. However, the importance of probiotic adhesion to mucus, low induction of respiratory infections in PMN, and low resistance to human serum-mediated killing may require further assessment by animal models and epidemiological data.

The "Toxicity" of Lactic Acid Bacteria

In the long history of thousands of years, food safety issues related to LAB were rare, and the incidence of related human diseases has been extremely low (excluding the *Enterococcus* and *Streptococcus* strains mentioned above). Safety assessment of LAB is also closely related to human scientific progress and technological development, so there are some hesitations when referring to terms such as "toxicity," "virulence factor," and "pathogenicity" related to LAB. The terms "toxicity" and "virulence factor" have been temporarily used in the following discussion before the emergence of a better explanation of the disease terminology caused by LAB. Even for *Enterococcus*, the concept of pathogenic factors is less precise than

those commonly recognized as Gram-positive pathogens such as *Botulinum*, *Staphylococcus aureus*, *Listeria*, and *Bacillus cereus* in foods. However, considerable progress has been made in the safety assessment of enterococci in recent years, such as the well-described factors associated with specific stages of enterococci infection (Franz and Holzapfel 2004). Johnson studied pathogenic strains at the specific stage that could cause infection and invade host tissues, resist the host's specific and non-specific defense system, cause pathological changes in the host, directly produce toxins, and indirectly cause inflammation (Johnson 1994). The virulence factors of enterococci involve all four stages mentioned above (Table 11.1). Franz et al. reviewed the mechanisms of these virulence factors in infection (Franz and Holzapfel 2004; Franz et al. 2003). It is noteworthy that Eaton et al. reported that enterococci isolated from food have one or more virulence factors, but the incidence of probiotic enterococcal virulence factors is significantly lower than that of food strains (Eaton and Gasson 2001; Franz et al. 2001). This suggests that *Enterococcus* carries virulent factors in food and poses a safety risk, especially for people with underlying diseases. To sum up, the European VRE (vancomycin-resistant *Enterococcus*) is likely to be transferred through the food chain, so for enterococcal risk strains with antibiotic resistance or carrying virulent factors, we should focus on the supervision of food transport routes.

Table 11.1 The virulence factors and toxic effects of enterococci (Franz et al. 2003)

Virulence factor	Related toxic phases
Polymer	Adhesion: adherence to eukaryotic cells or promotion of colonization
	Invasion: invasion of eukaryotic cells
	Promoting migration: adhesion to extracellular matrix proteins
	Evading host immune response: enhancing immune cell survival
Cell lysin	Release of eukaryotic toxin
	Evasion of host immune response: lysis of immune cells
Gelatinase	Displacement: hydrolysis of various biological peptides, such as collagen and fibrin.
	Evasion of host innate immune response: hydrolysis of antimicrobial peptides
Enterococci surface protein	Evading host immune response: presenting bacterial surface recognition and adhesion matrix molecular characteristics
Enterococcus faecium or *Enterococcus faecium* collagen adhesin	Promoting migration: adhesion of extracellular matrix proteins
	Evading host immune response: presenting bacterial surface recognition and adhesion matrix molecular characteristics
Hyaluronidase	Displacement: degrading hyaluronic acid (a major extracellular matrix)

Because non-enterococcal LAB have a long history of food safety, its possibility of toxicity is very low, and it is difficult to find "virulence factors" in them. Although it has been reported that some strains, such as *Weissella confusa*, have alpha-hemolytic phenotypes and some *L. lactis* in dairy products have been confirmed to have a hemolysin III gene (Olano et al. 2001; Bolotin et al. 1999), the effects of these hemolytic characteristics are negligible, because no infection induced by these hemolytic characteristics was reported so far. Recently, many reports were about further sequencing of LAB genome; the potential virulence exists in their genome informations (Bolotin et al. 1999; Kwon et al. 2015; Kwak et al. 2015; Yu et al. 2012; Pridmore et al. 2004; Kleerebezem et al. 2003). The genome of probiotic *L. yoelii* NCC 533 showed a protein with 50% amino acid sequence similarity to the IgA protease of pathogenic *Streptococcus*, which may be related to the hydrolysis of extracellular proteins or adhesion to mucosal surfaces (Pridmore et al. 2004). IgA protease can prevent bacteria trapped in mucous layer and is thought to be immune from host immune defense. However, the IgA protease can only be regarded as an attribute of the probiotic strain without obvious virulence factors. Therefore, the role of IgA protease may be related to the colonization of probiotics in the gastrointestinal tract, rather than toxicity.

Vesterlund et al. studied the presumed risk factors in feces, blood, and *Lactobacillus* strains (Vesterlund et al. 2007). The adhesion of extracellular proteins and mucus, hemolysis, the ability of monocytes in peripheral blood to avoid respiratory burst, and the tolerance to human serum were studied. For the test strains, adhesion to collagen, fibrinogen, and mucus was strain-specific, and no significant difference was observed between blood, feces, and beneficial biomass. This study did not find any clear virulence factors in *Lactobacillus*.

11.1.1.2 Safety Events and Current Opinions of Lactic Acid Bacteria

For special cases with relatively low immunity or congenital infections, ingestion of specific lactic acid bacteria in a certain period of time would induce immunoreaction, including hypersensitivity reactions, so special individuals should be cautious when selecting lactic acid bacteria products. In the 1990s, Schwab et al. reported that the ingestion of lactic acid bacteria through non-oral routes would cause immune responses and cause joint pain and complications (Schwab 1993). Studies have shown that the symptoms of these over-immune reactions are mediated by cytokine secreted by lactic acid bacteria.

Cases of probiotic translocation have been reported in the past decade. Kunz et al. observed two cases of LGG bacteremia during the treatment with lactobacilli (Kunz et al. 2004). In 2005, another case of LGG bacteremia was associated with the use of probiotics. In addition, Land et al. demonstrated that lactobacilli sepsis induced by ingestion of probiotics in two pediatric patients is not easy to find out (Land et al. 2005). Similarly, LeDoux et al. reported *L. acidophilus* bacteremia happened after ingesting probiotics (LeDoux et al. 2006). These cases have sounded the alarm to us. Although all probiotics that caused bacteremia were reported in patients,

such as short-term bowel syndrome, and AIDS or premature babies, it is important to consider the risk of some probiotics causing bacterial translocations, sepsis, and bacteremia. In a systematic review of the safety of probiotics in patients with nutritional support, Whelan et al. reported that 32 patients in 20 adverse events were infected with LGG or *Saccharomyces boulardii* (Whelan 2010). They concluded that certain probiotic products (single or combination) may increase the risk of complications in a particular patient population.

A 5-year-old patient developed D-lactic acidosis after bowel resection. His condition improved after the treatment, but acidosis was followed by intake of mixed LAB supplement. It may be due to over-colonization of the genus producing lactic acid in the intestinal tract (Ku 2006). It has been reported that the intake of LAB caused methemoglobinemia in infants (Nitrate 1995). This is because LAB in the mouth have strong nitroreductase activity, which will cause the nitrate in the food to form nitrite, and the lesion occurs in the acidic stomach environment. At the same time, as a precursor of nitrosamines, the presence of nitrite may also have a hidden danger of cancer. The lactic acid bacteria in the intestine have strong amino decarboxylase activity, which can act on free amino acids to convert them into biogenic amines, leading to food poisoning disorders such as nausea and vomiting.

In addition, Besselink et al. reported that probiotic ingestion has increased the mortality rate of severe acute pancreatitis, and probiotics are not administered in this category of patients (Besselink et al. 2008a). In recent years, some consumers have developed bacteremia after consuming large amounts of dairy products containing *Lactobacillus casei*. These cases demonstrate that the safety of probiotics is not just an accidental isolated event, but is always present in clinical treatment. The bacterial translocation caused by these probiotic strains and subsequent cases of sepsis or endocarditis are a warning to us and emphasize the need to investigate the translocation capacity of probiotics and take it as an aspect of safety evaluation.

Combined with these events, probiotics cannot be considered to be harmless adjuncts to enteral nutrition, especially in critically ill patients or patients at risk for nonocclusive mesenteric ischemia. The probiotic interventions are not recommended in specific patient populations, such as those with severe acute pancreatitis.

11.1.1.3 Development of Lactic Acid Bacteria Safety Evaluation

To date, clinical cases of infection caused by lactic acid bacteria have rarely been found in healthy people. In most cases of clinical infection caused by lactic acid bacteria, the lactic acid bacteria that cause infection are almost exclusively derived from the free bacterial flora in patients. In 1938, the first case of endocarditis caused by *Lactobacillus* infection occurred. In the following 55 years, 53 cases of endocardial inflammation caused by *Lactobacillus* were found, indicating the incidence was very low. *Lactobacillus* is widely distributed in humans as a common commensal flora. Compared with another type of commensal flora such as *Streptococcus* or *Staphylococcus*, the ratio of lactobacilli to endocardial inflammation is very low (0.05%).

In September 1993, the International Association of Microbiology Societies (IUMS) believed that probiotics including lactic acid bacteria had pathogenic risks, such as invasive or toxic arguments lacking food or clinical case evidence support. However, probiotic productions include not only bifidobacteria but also enterococci, propionic acid bacteria, or brewer's yeast; therefore it is necessary to confirm the safety of the relevant edible supplement strains.

The status of probiotic LAB (including *Lactobacillus* and *Bifidobacteria*) is generally recognized as safe (GRAS) in four aspects: human trials, animal models, in vitro tests, and market research on probiotic products. The human experiment was mainly based on 143 human clinical trials conducted from 1961 to 1998. It was confirmed that oral probiotic lactic acid bacteria had no adverse side effects, and 7526 subjects all responded well, thus confirming its safety. In animal experiments, oral or injection probiotics did not cause suppurative infection, acute toxicity, bacteremia, etc. in mice, rats, and guinea pig models. Not only that, probiotics can significantly extend the life-span in animal experiments. At the same time, lactic acid bacteria were not invasive to human lymphoma cells in vitro.

Although safety incidents rarely occur, with the increase of bacterial resistance and the continuous discovery of pathogenic factors in enterococci, people are paying more attention to the safety of probiotics. In the European Union seminar in early 2000, the safety of LAB was discussed, and the risk of infection by lactic acid bacteria was low except for *Enterococcus*. However, considering that the probiotics currently used are separated from different infectious parts, the monitoring needs for commercial lactic acid bacteria such as *L. rhamnosus* are continued, and the safety of probiotics should always be kept in the industrial application.

Internationally, there are different ways and various categories of regulations that limit the use of lactic acid bacteria in food. There are differences between countries, which are not necessarily related to strain's function. According to the Federal Food, Drug, and Cosmetic Act in 1958, the cultured microorganisms used in the United States are defined as "food additives," and new products are subject to safety standards before they are launched. However, according to Section 210 of the act, certain substances are expressly excluded by the US Food and Drug Administration (FDA), and the substances of the generally recognized as safe (GRAS) are specifically mentioned. Therefore, the FDA also lists specific familiar microorganisms that are considered safe. Although this type is not GRAS, they are microorganisms traditionally used in the food industry.

11.1.2 Safety and Risk Status of Different Uses of Lactic Acid Bacteria

The current reports on the harmful effects of probiotic strains are less, while the demand for new strains is increasing, and there is a huge market and industrial production potential. Therefore, we cannot assume that the new strains have the same safety as the traditional strains that have been tested. Domestic research on the

safety of lactic acid bacteria focuses on two aspects: one is the probiotic mechanism and functional research related to lactic acid bacteria; the other is the potential ability of lactic acid bacteria as a safety strain to become a genetic engineering carrier. Although research on intestinal microbes and other lactic acid bacteria has become a hot topic in recent years, there are few studies on the safety of lactic acid bacteria, and it showed that domestic safety awareness and attention on lactic acid bacteria are low, which still stays in the "very safe" evaluation stage, indicating that there is insufficient understanding of the potential risks and hazards of lactic acid bacteria from consumers to researchers to food safety departments and lack of crisis awareness. At some international seminars about lactic acid bacteria, the safety issues of related probiotics have also been mentioned or fully valued. Researchers, especially enterprises and R&D institutions, have invested more energy into the functional research of lactic acid bacteria, and the safety problems are greatly neglected.

11.1.2.1 Safety Status of Lactic Acid Bacteria in Food

Status of Antibiotic Resistance Genes in Lactic Acid Bacteria

Recently, it has been reported that many intestinal bacteria have antibiotic resistance genes. Masco et al. found that some bifidobacteria isolated from human, animal, and probiotic products have tetracycline resistance activity, and the *tetW* gene is responsible for drug resistance expression in all strains (Masco et al. 2006). Aires et al. reported that bifidobacteria isolated from humans contain 26% and 7% of the *tetW* and *tetM* genes, indicating that many bacteria in our gut acquired antibiotic resistance, such as *tetW*, and having the risk that the antibiotic resistance gene has been transferred to harmful bacteria (Aires et al. 2007). Liu and Pop created a database of antibiotic resistance genes (Liu and Pop 2009), and we continue to expand the resistance gene library of probiotics including bifidobacteria and lactobacilli in this book. Some antibiotics such as *tetW*, *tetC*, *tetL*, and *cata*-1 are expressed in some strains of *Bifidobacterium* and *Lactobacillus* (Table 11.2). The *tetW* gene is

Table 11.2 List of antibiotic resistance genes carried by bifidobacteria in the antibiotic resistance gene database[a]

Antibiotic type	Drug resistance gene	Bifidobacterium listed in the database	Lactobacillus listed in the database
Tetracycline	*tetW*	*B. longum* subsp. *infantis*, *B. bifidum*, *B. animalis* subsp. *lactis*	*L. reuteri*, *L. johnsonii*
Tetracycline	*tetC*	*B. thermophilum*, *Bifidobacterium* spp., *B. bifidum*	*L. reuteri, L. paracasei*
Tetracycline	*tetL*	*B. thermophilum*	*L. sakei*
Chloramphenicol	*cata-1*	*B. adolescentis*	
Chloramphenicol	*ermX*	*B. thermophilum*	

[a]The database of antibiotic resistance genes: http://ardb.cbcb.umd.edu/index.html

present in various bifidobacteria such as *Bifidobacterium longum*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *infantis*, and *Bifidobacterium thermophilus*. Therefore, various studies have shown that many bifidobacteria and lactobacilli in the human gut have already carried antibiotic resistance genes and that this antibiotic resistance may be present in pathogenic bacteria, posing a potential threat.

Some reports have revealed the source of antibiotic resistance and how these genes are transmitted to bifidobacteria and other bacteria and studied the antibiotic susceptibility in probiotic products containing bifidobacteria in the Japanese market. In the report, *Bifidobacterium* spp. are isolated from various probiotic products, including *Bifidobacterium longum* subsp., *Bifidobacterium breve*, and *Bifidobacterium animalis* subsp. *lactis*, in which all *Bifidobacterium animalis* subsp. *lactis*, have tetracycline resistance and carry the *tetW* gene. Kastner et al. also found that *B. lactis* isolated from probiotic products contained the *tetW* gene (Duran et al. 2012; Kastner et al. 2006). In addition, Gueimonde et al. reported that all *B. animalis* subsp. *lactis* strains isolated from products including yogurt, fermented food, or probiotic supplements carry tetracycline resistance gene *tetW* (Gueimonde et al. 2010). These findings indicate that there are many commercial products of bifidobacteria probiotics containing the *tetW* gene, and the existence of the gene *tetW* is the minimum requirement of safety assessment on the selection of probiotic *Bifidobacterium* strains.

Safety Status of Lactic Acid Bacteria Products Infected with Human Body

Because the usage amount of lactic acid bacteria is very large, the safety evaluation of lactic acid bacteria is very important. The safety of some probiotics with a long history is clear. For a long time in the past, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus thermophilus* have been used in food processing. There was a long history that people consumed foods containing lactic acid bacteria or their metabolites (Ishibashi and Yamazaki 2001; Yan et al. 2015; Doron and Snydman 2015). Until now, the safety of these bacteria has not been questioned, and reports of the harmful effects of these bacteria are extremely rare (except for pathogenic enterococci and streptococci). The infection cases caused by *Lactobacillus* and *Bifidobacterium* are very rare, and the probability of causing endocarditis or bacteremia is only 0.05–0.40%. But if early diagnosis and appropriate treatment are not performed, the overall mortality rate is high, close to 25% (Cannon et al. 2005). It has been reported that *Leuconostoc* causes less than 0.01% of bacteremia cases, and lactic acid bacteria are also associated with epidemic diseases in specific hospitals in Spain or India (Bou et al. 2008; Taneja et al. 2005). *Enterococcus* is a major exception in lactic acid bacteria (without pathogenic streptococci), and its probability of causing bacteremia is 5–15% (Aguirre and Collins 1993; Wang and Wang 2014; Gasser 1994; Salminen et al. 1998; Saxelin et al. 1996).

Most of the lactic acid bacteria that cause infection in the population belong to *Enterococcus faecalis* and *Enterococcus faecium* (Johnson 1994; Franz et al. 2003;

Oguntoyinbo and Okueso 2013; Valenzuela et al. 2009; Murray 1990), but other lactic acid bacteria, including *L. rhamnosus*, *L. acidophilus*, *L. paracasei*, *L. casei*, *L. curvatus*, *L. sphaericus*, *L. salivarius*, *Leuconostoc lactis*, *Leuconostoc citreum*, *Leuconostoc pseudomesenteroides*, *Pediococcus acidilactici*, and have also been reported in human infections. Cannon et al. (2005) reported that 241 cases of endocarditis and bacteremia were caused by *Lactobacillus* between 1950 and 2003. *Lactobacillus casei* (35.7%) and *Lactobacillus rhamnosus* (22.9%) are the most common strains causing infective lactobacilli and endocarditis. In general, the vast majority of patients infected with lactic acid bacteria have a potential disease, which make them more susceptible to infection. Age and pregnancy are not risk factors for lactic acid bacteria infection. For enterococci, infective endocarditis is prone to occur in patients with underlying heart disease. Risk factors of endocarditis include genitourinary diseases, abortion, or urinary tract infections. Risk factors of bacteremia caused by *Enterococcus* include underlying diseases, urethral or vascular bypass, surgery, severe burns, multiple trauma, or antibiotic treatment (Franz et al. 2003; Morandi et al. 2013; Fortina et al. 2008). For *Pediococcus* infections, there is a risk of the following underlying disease such as diabetes, tuberculosis, vascular complications, burns, thyroid hyperactivity, abdominal surgery, stomach tube feeding (whether pregnant or not).

The individuals with low immunity are generally more susceptible to be infected. However, consumption of probiotics (lactic acid bacteria) does not increase the infection chance in these populations (Borriello et al. 2003). In contrast, probiotics are widely used in the clinic for the treatment of diseases. Probiotic lactobacillus is reported to treat children with severe underlying diseases and diarrhea (Land et al. 2005). The probiotic strain *L. rhamnosus* is also used to treat diarrhea caused by invasive diseases. Besselink et al. (2008a, b) and van Baal et al. (2011) found that oral administration probiotics containing four lactic acid bacteria and two *Bifidobacterium longum* can improve therapy efficacy in severe acute pancreatitis (van Baal et al. 2011; Besselink et al. 2008b). Nevertheless, probiotic prevention does not reduce the risk of infection complications and is even associated with an increased risk of mortality.

11.1.2.2 Safety Status of Clinical Medicinal Lactic Acid Bacteria

With the development of microecology research on lactic acid bacteria, there is growing concern on the health of gut microbes, and related microecological preparations are also emerging. However, there are still security concerns on using lactobacillus and other bacterial formulations in health care and clinical care. Common medical probiotic preparations generally include a single live bacterial preparation, such as bifidobacterium live bacteria capsule, lactasinum biofermin, etc.; multi-bacterial combined preparations, such as bifidobacteria triple live bacteria capsules; and dead bacteria preparations, such as oral lactobacillus dispersion. Besides, functional exploration of probiotics is not limited to the intestines; other organs, such as the skin, vagina, oral cavity, etc., are also well studied and have been clinically applied.

At present, probiotic lactic acid bacteria applied to clinical or health-care drugs are continuously supplemented and being developed; the microecological drugs that have obtained the drug approval number in China are mainly active bacteria probiotic preparations. These probiotics are mainly lactic acid bacteria and some *Bacillus* and physiological fungi. In general, the normal gut microbes consist of three types of bacteria: resident bacteria, symbiotic bacteria, and passing bacteria. Among them, the resident bacteria are relatively stable, indispensable, and low in immunogenicity and mostly are anaerobic bacteria; the symbiotic bacteria rely on the original bacteria to exert physiological functions, which cover most lactic acid bacteria in the gut, while passing bacteria, including nonpathogenic bacteria and conditional pathogens, can colonize in the host intestinal and cause disease when the immune function is impaired or the resident bacteria are disordered. It can be seen that the drug lactic acid bacteria as gut microecological preparation can reach the target organ through the host digestive tract barrier, colonize the digestive tract in a certain period of time, and exert a physiological action and can be metabolized naturally from the host. Although there is great potential of lactobacillus in domestic medicine, the related research and development is still in start-up stage, and it is urgent to introduce and implement relevant medical safety regulations. How to rationally use lactobacillus in clinical and other medical medicines and what safety problems exist in probiotic products have not been solved.

Lactic acid bacteria are not the main drug in clinical medicinal. High school medical practice in a small Canada town showed that only 31% of resident had clinical knowledge of probiotics, while 24% believed that probiotics do not work in medical practice (Edmunds 2001). However, 76% of physicians believe that probiotics are very useful in their clinical practice; the potential market and the lack of relevant experience in this area are worrisome. Many health-care professionals, such as hospital practitioners, physiotherapists, massage therapists, and herbalists, routinely use products containing lactobacilli, bifidobacteria, or other probiotics. However, due to the limitations of relevant training, doctors may not be able to access relevant evaluations and cannot discuss the advantages and disadvantages of the so-called nontraditional, complementary, alternative medicines, which contain probiotics. The government plans to conduct policy to manage such drugs separately from other categories; it is difficult for small businesses to find suitable probiotic-related regulations and drug licenses. At the same time, when doctors use or recommend these drugs, they may not able to provide relevant tests to show these drugs have no clinical side effects and are reliable, since it is difficult to ensure that the large number of strains used in clinical practice has been sufficiently tested under current research fund conditions.

There are many factors that have prompted doctors to test and evaluate probiotic strains and other drug substitutes, including fluctuations in the multidrug resistance genes carried by pathogens, increased demand for natural medicines, and some probiotic effectiveness evidenced by scientific and clinical experiments. Governments and research institutions should promote relevant regulations and guidance as soon as possible to ensure the reliability of clinical application strains. Without such a product identification model, doctors will know very little about the probiotics they provide to patients, which is very unfavorable for clinical use. However, the current

situation is still severe; the analysis of probiotic strains shows that the strains currently available for drugs, food, or dietary supplements are difficult to meet clinical medicinal standards.

11.1.2.3 Safety Status of Lactic Acid Bacteria in Feed

Feed enters the human food chain through animal products, so its safety is directly related to food safety and human health. In the 1980s, the successive occurrence of major feed safety incidents caused widespread international concern, and the feed safety issues caused the international wide attention, and it was considered as important as the food safety issues. Microorganisms were added to feed as an additive and were generally considered to be a new green additive that is naturally safe and not harmful to health. With the development of economy and technology, people have higher requirements for health and safety, and relevant regulations and requirements in the process of applying microorganisms to feed are needed to ensure their development.

There are few reports on the safety of lactic acid bacteria supplemented in the feed in China, which cannot fully explain the safety of lactic acid bacteria. Existing safety problems cannot be ignored though we started late, and relevant research has not been carried out by the government. Current scientific research are mostly based on foreign research and analysis, and research experiment indicators on the feed production mainly focused on daily gain, feed conversion rate, mortality, and morbidity, but there are few indicators around safety. Research on microbial strains can be based on several questions in key steps in the production process: screening the source of the strain, the strain variation during the breeding process, the mutual exclusion between the strains, the toxicology of the finished feed, and epidemiological analysis. In addition, the amount of microorganism supplemented in feed should be analogous to that of common chemical drugs and antibiotics, and it can have a beneficial effect only when the corresponding amount of additives were attained, that is, the number of viable bacteria reaches a certain cfu/ml and then the formulated product meets the corresponding requirements. However, there is almost no regulation on the content of lactic acid bacteria in the products for feed addition at present, so further research and improvement of relevant specifications are needed in these aspects.

11.2 Evaluation Methods and Principles of Lactic Acid Bacteria Safety

11.2.1 General Safety Evaluation Methods for Lactic Acid Bacteria

Recently, with the development and prevalence of lactic acid bacteria and related products, some serious side effects including the sepsis caused by bacterial translocation and the horizontal transfer of antibiotic resistance genes can happen, and the

safety of probiotics, especially a large proportion of lactic acid bacteria, has been attracting more and more attention. In response to these safety concerns, manufacturers must demonstrate the safety of probiotics on species levels, as experimental studies do not demonstrate that all probiotic strains have the same properties. On one hand, probiotics with antibiotic resistance genes should not be used in industrial production in order to prevent the outbreak of pathogenic bacteria with antibiotic resistance, since current studies cannot prove whether the possible gene of horizontal transfer is located on chromosome DNA. On the other hand, there is a need to improve hygiene standards in the production process to prevent probiotics from being contaminated by pathogenic bacteria or allergens to ensure the safety of the probiotics.

The following discussion will focus on the most common safety issues of lactic acid bacteria involving the drug resistance, translocation, and production of harmful metabolites of the bacteria. Courvalin reviewed the safety of the probiotics on antibiotic resistance and proposed an algorithm based on the antibiotic resistance for antibiotic selection of probiotics (Courvalin 2006). Based on this method, we can determine whether a strain is suitable for probiotics based on the following few steps. Firstly, we need to know the phenotype of antibiotic resistance, and only the strain with no resistance to specific antibiotics for probiotic and the strain with resistance to specific antibiotics can be confirmed safe when the antibiotic resistance genes are conserved. Secondly, if the transferability of the bacteria cannot be assessed, we need to determine whether the antibiotic gene is available in consideration of the gene transferred to other bacteria, and the strain can be safe only when the genes are proved to be conserved in chromosomes. For example, if the DNA fragment contains homologous cognate of family genes, the strain is credible. This rule is not difficult to understand and can make the whole study extremely meaningful.

In recent years, with the wide spread of many antibiotic resistance genes, concerns have been growing that probiotics carry pathogenic genes and cause pathogenic bacteria to have antibiotic resistance genes. Probiotics carrying antibiotic resistance genes cannot be used in food chains and feeding areas as recommended by FAO/WHO or EFSA. To prevent the spread of antibiotic resistance genes in the environment (including our gut), all probiotic product manufacturers need to review the product from the perspective of antibiotic resistance and select the appropriate probiotics according to the rules set by Courvalin (Courvalin 2006).

11.2.1.1 Evaluation of Drug Resistance of Lactic Acid Bacteria

Overview of Drug Resistance and Drug Resistance Genes of Lactic Acid Bacteria

Lactic acid bacteria strains isolated from feces of human infected by diseases, particularly *Enterococcus*, are known to be resistant to various antibiotics (Klare et al. 1995a). *Enterococcus* may be born with an intrinsic resistance gene, and the

resistance gene is located on the chromosome or acquired, and its resistance gene is located in the plasmid or transposon (Murray 1990; Klare et al. 1995a; Clewell 1990). Intrinsic antibiotic resistance includes antibiotics of cephalosporin, low concentration of β-lactam, sulfonamide, low concentration of clindamycin and aminoglycoside antibiotics, and acquired resistance includes antibiotics of chloramphenicol, erythromycin, and high horizontal clindamycin, aminoglycoside antibiotics, tetracycline, high concentrations of beta-lactams, quinolones, and glycopeptides such as vancomycin (Murray 1990; Klare et al. 1995a; Leclercq 1997). The resistance to vancomycin is especially a concern for it is considered to be the last treatment for infectious diseases caused by multidrug-resistant enterococci. In the mid-1990s, anti-vancomycin enterococci (VRE) in Europe proved to be derived from farm animals and probably due to the extensive use of the glycopeptide antibiotic vancomycin (Bjorkeng et al. 2011; Klare et al. 1995b). VRE have also been shown to be isolated from various farm animals, which are the main cause of VRE (Klare et al. 1995b; McDonald et al. 1997). Therefore, in 1997, the EU prohibited the use of vancomycin in animal husbandry. In 1999, the United States approved the use of streptavidin B/A-quinapoddine-dafopridine (Synercid®, King's Pharmaceuticals, Bristol, TN) for the treatment of VRE. Jensen et al. reported that anti-streptavidin strains were transferred from farm animals to farmers, indicating that enterococci-resistant strains are increasing (Jensen et al. 2000). Recently, new antibiotics such as linezolid and daptomycin have been successfully used in the treatment of VRE; however, the emergence of drug-resistant strains seems to be only a matter of time, and multidrug-resistant enterococci strains with these antibiotics are constantly emerging.

Non-enterococci can be obtained from medicine and food, some of which are inherently resistant and the others are transferable. *Leuconostoc*, *Pediococcus*, and some *Lactobacillus* such as *L. rhamnosus*, *L. paracasei*, *L. plantarum*, and *L. reuteri* are inherently resistant to vancomycin, while most *Lactococcus* and *L. acidophilus* are susceptible to it (Klare et al. 2007; Ammor et al. 2007; Delgado et al. 2005; Danielsen and Wind 2003). Klein et al. proposed that the resistance of *Leuconostoc*, *Pediococcus*, and *Lactobacillus* to vancomycin was due to the D-alanine-D-lactic acid structure in its peptidoglycan (Klein et al. 2000).

Most of the *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Pediococcus* are resistant to metronidazole because they lack the hydrogenase activity (Ammor et al. 2007). On the one hand, most *Lactobacillus*, *Pediococcus*, and *Leuconostoc* can tolerate high concentrations of cephalosporins. On the other hand, *Lactobacillus* and *Lactococcus* are sensitive to penicillin and β-lactam antibiotics but resistant to cephalosporin. And the resistance of *Streptococcus thermophilus* to penicillin G and ampicillin is highly variable (Ammor et al. 2007; Temmerman et al. 2003; Katla et al. 2001). Penicillin G and imipenem, a β-lactam antibiotic, are generally active against *Pediococcus*; however, some reports suggested that some *Pediococcus* are resistant to β-lactam (Ammor et al. 2007).

Lactobacillus, *Leuconostoc*, and *Lactococcus* are generally intolerant to antibiotics that inhibit protein synthesis, such as chloramphenicol, erythromycin, clindamycin, and tetracycline antibiotics, but are resistant to aminoglycoside antibi-

otics such as neomycin, kanamycin, streptomycin, and gentamicin tolerance (Ammor et al. 2007; Delgado et al. 2005; Danielsen and Wind 2003; Temmerman et al. 2003; Katla et al. 2001; Hummel et al. 2007; Gevers et al. 2003). Some *Lactobacillus*, *Lactococcus*, and *Pediococcus* strains have been shown to be highly resistant to chloramphenicol, clindamycin, streptomycin, erythromycin, and tetracycline (Klare et al. 2007; Ammor et al. 2007; Temmerman et al. 2003). In many cases, antibiotic resistance is attributed to the presence of drug-resistant genes, for instance, *Lactobacillus plantarum* (Ahn et al. 1992) and *Lactobacillus reuteri* (Lin et al. 1996) have toxin acetyltransferase (*CAT*) gene. Moreover, *erm(B)* and *erm(T)* genes encoding erythromycin are present in *Lactobacillus*, *Lactococcus*, and *Pediococcus* (Ammor et al. 2007; Florez et al. 2006; Stroman et al. 2003). The resistance of macrolide antibiotics is related to the mutation of 23S RNA gene locus from A to G, and the resistance of lactic acid bacteria to tetracycline is related to tetracycline resistance genes such as *tet (K*, *M*, *O*, *Q*, *S*, *W)* (Ammor et al. 2007; Gevers et al. 2003; Huys et al. 2004). Rojo-Bezares et al. found the tetracycline resistance gene *tet (L)* in *Pediococcus parvulus* from wine (Rojo-Bezares et al. 2006). At the same time, it has been reported that aminoglycoside antibiotic resistance genes such as *aac(6′)le-aph(2″) la*, *aac(6′)-aph(2″)*, *ant(6) and aph(3′)-llla* exist in some *Lactobacillus* and *Lactococcus*. Studies also showed that multiple drug transporters (*Mdt*, *LmrP*) are associated with resistance of lactobacilli to antibiotics such as streptavidin, tetracycline, and macrolides (Putman et al. 2001; Perreten et al. 2001). Lactobacilli are generally resistant to most nucleic acid synthesis inhibitors such as enrofloxacin, pefloxacin, norfloxacin, nalidixic acid, sulfonamide, trimethoprim, and metronidazole, and their drug resistance is intrinsic resistance rather than acquired resistance (Ammor et al. 2007; Han et al. 2015; Charteris et al. 1998).

In conclusion, it is recommended to use β-lactam (penicillin or ampicillin) and aminoglycosides (usually gentamicin) for synergistic treatment in the case of *Lactobacillus* infection, and erythromycin or clindamycin is available as an optional adjuvant treatment (Cannon et al. 2005; Danielsen et al. 2007).

Risk Assessment of Lactic Acid Bacteria Carrying Antibiotic Resistance Genes

Teuber et al. warned the presence of antibiotic resistance genes in the human gut microbiota and food chain (Besselink et al. 2008a). Unfortunately, despite these warnings, many studies have shown that antibiotic resistance genes remain increased in the past 10 years, and *Enterobacteriaceae* carrying the antibiotic resistance gene *NDM-1* has also aroused great concern. Since the transfer of antibiotic resistance genes to pathogenic bacteria may raise serious problems, careful investigation and ongoing monitoring of the potential drug-resistant gene transfer in the environment must be conducted.

Probiotic products contain yogurt, infant formula, cheese, and dietary supplements, which may contain a wide variety of bacterial species (Phillips et al. 2006; Champagne et al. 2005; Vinderola et al. 2000). A large number of consumers con-

sume a significant amount of probiotics each year, and these probiotic strains can interact very closely with many high-density gut microbiota. Therefore, antibiotic resistance genes can be transferred to pathogens or gut microbiota by probiotic bacteria, which can increase the risk of widespread spread of these genes or bacteria in our gut or environment.

Some studies indicate that the sequence of tetracycline resistance gene *tetW* in the tetracycline-resistant *Clostridium difficile* isolated from humans was found to have 99% homologous similarity to that of *B. longum* F8 strain gene sequence (Roberts et al. 2015; Spigaglia et al. 2008). This finding suggests that the *tetW* gene may be derived from a gene exchange between the two strains (which is very unlikely) or derived from the microbial source sequence gene in the same gut.

Study also suggests that the *tetW* gene may spread through horizontal gene transfer. In fact, Jacobsen et al. have demonstrated horizontal gene transfer from *Lactobacillus plantarum* to *Enterococcus faecalis* in the gut of sterile mice (Roberts et al. 2015; Spigaglia et al. 2008). These experiments showed that the human intestinal tract may provide a good environment for the horizontal transmission of gene levels (including antibiotic resistance genes). For these reasons, whether antibiotic resistance genes presented in probiotic strains need to be explored and resolved.

The Measurement of Lactic Acid Bacteria Resistance

Since there is no unified method and standard for establishing drug resistance tests for lactic acid bacteria, it is difficult to evaluate the drug resistance genes of lactic acid bacteria. Lactic acid bacteria are different from pathogenic bacteria and are not the main cause of disease in the body. In addition, many lactic acid bacteria have high nutritional requirements, so they are only suitable for living in nutritious environment. This has led to routine drug resistance test media, such as Mueller-Hinton or Iso-Sensitest media, which are often not suitable for drug resistance testing of *Lactobacillus*, *Leuconostoc*, or *Pediococcus* (Klare et al. 2007). Herra et al. pointed out that the growth of *Lactobacillus* was inhibited when Wilkens-Chalgren agar medium recommended by the National Committee for Clinical Laboratory Standards (NCCLS) was used to determine anaerobic resistance (Herra et al. 1995). However, if 5% horse blood is contained in the Wilkens-Chalgren agar medium, the *Lactobacillus* will grow well. Besides, the choice of medium in the resistance test can greatly affect the minimum inhibitory concentration (MIC). For the selection of the medium, the nutrition in the medium is best suited to test bacterial growth. Then, the medium has a suitable gel structure to facilitate the diffusion of antibiotics and repeated experiments. Finally, known or unknown components of the medium do not interact with the antibiotics or have a little interaction (Huys et al. 2002). It is well known that the results of the test will vary with changes in the concentration of cations and essential nutrients such as thymine and folic acid in the medium. Therefore, in addition to the composition of the medium, the number of inoculation, the temperature of the culture, the composition of the gas, and the culture period may affect the results of the strain resistance test.

In order to solve the problem of test medium standardization, Klare et al. modified a medium to test the susceptibility of lactic acid bacteria (Klare et al. 2007). It is a mixture of 90% Iso-Sensitest medium and 10% MRS medium. It can be added or not added with L-cysteine when testing the susceptibility of lactic acid bacteria. This medium can provide nutrient-rich lactic acid bacteria (not including *Enterococcus*) to grow, and the MIC of different lactic acid bacteria can be well detected. The results of Klare et al. showed that the MIC value was similar to the Mueller-Hinton medium of which cation concentration was adjusted and was supplemented with horse blood (Klare et al. 2005). Mueller-Hinton is a medium recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2007 for testing the complex nutritional microbial resistance. Tosi et al. developed a *S. thermophilus* test medium (SSM) formulation for the specific detection of resistance to *S. thermophilus* (Tosi et al. 2007). This medium can well test the atypical tetracycline resistance of the strain. The SSM medium was formulated with 90% Iso-Sensitest medium and 10% M17 medium supplemented with 0.5% (w/v) lactose.

Lactobacillaceae family contains a rich variety of genus contains plenty of species, it is therefore difficult to choose a MIC threshold to separate resistant and nonresistant strains. In brief, a resistance threshold can be set on this genus when most strains in a genus have similar resistance to an antibiotic. However, for most lactic acid bacteria, this setting is too simple, because different species have great differences in resistance to different antibiotics. The European Commission's Committee on Animal Nutrition provides a list of drug resistance thresholds for lactic acid bacteria (not including *Enterococcus*), which is an immature list because it only includes thresholds for *Pediococcus*- and *Lactobacillus*-related genera and does not consider the difference between species. Danielsen and Wind believed that there are still many shortcomings in using this method to study the drug resistance of lactic acid bacteria, because many strains have strong drug resistance, and their threshold will be much higher compared to those drug sensitivity strains (Danielsen and Wind 2003). The Scientific Commission for Animal Nutrition (SCAN) gives a MIC threshold concentration of 1 μg/ml for gentamicin; however, Danielsen considered that this value is too low, and it is recommended to use the threshold of 128 μg/ml for the resistance of *L. paracasei*, *L. plantarum*, *L. sinensis*, *L. pentosus*, *L. brevis*, and *L. rhamnosus* and use a threshold of 256 μg/ml for testing for *L. acidophilus* resistance. At the same time, Danielsen also believes that the streptomycin threshold of 16 μg/ml set by SCAN is too low and should be adjusted to >256 μg/ml for all lactic acid bacteria. In addition, they believed that the 4 μg/ml erythromycin threshold recommended by SCAN is too high, leading to the undetectable resistance of certain lactobacilli. For the erythromycin threshold, the recommended amount is 1 μg/ml for *L. acidophilus*, *L. sphaericus*, and *L. curvus*, 2 μg/ml for *L. paracasei* and *L. rhamnosus*, and 4 μg/ml for *L. plantarum* and *L. pentosus*. In 2005, the Animal Feed Additives and Products Group (FEEDA) gave a threshold table for 13 antibiotics for lactic acid bacteria that can distinguish between homolactic fermentation and heterolactic fermentation (*Lactobacillus plantarum*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus lactis*, and *Streptococcus thermophilus*).

Klare et al. studied the resistance of 16 antibiotics to different lactic acid bacteria strains, including *Lactobacillus plantarum*, *Pediococcus*, and *Lactococcus* (n = 473) (Klare et al. 2007). They proposed the experimental epidemiological cutoff (ECOFF) to describe the innate or acquired drug resistance of the strain. Klare et al. determined the ECOFF values of 12 lactic acid bacteria against different antibiotics, respectively. The strains are including *Lactobacillus pentosus*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lactobacillus delbrueckii*, and *Lactobacillus reuteri*. Therefore, the MIC threshold has a clear understanding. As predicted by the safety eligibility presumption, the MIC threshold can help us to determine the drug resistance of the strain and filter the strain with high resistance, to study its drug resistance gene and the transformability of this gene.

11.2.1.2 Evaluation of Lactic Acid Bacteria Translocation and Migration

Safety Testing of Lactic Acid Bacteria Migration Ability

In conjunction with the safety studies of the previous sections, in order to confirm the safety of probiotics associated with bacterial migration or sepsis, the following studies must be performed:

1. Viscolytic activity
2. Bacterial translocation ability in traditional animal models
3. Intestinal surface structure in animal models
4. Bacterial translocation ability in immunocompromised animal models
5. Infection activity after bacterial translocation
6. Clinical study of healthy people
7. Clinical study of susceptible people

Probiotics are usually consumed as food, and all people have the opportunity to consume probiotic products, including susceptible people. And most people get the expected health benefits after taking probiotics. However, it is necessary to confirm the validity of the probiotic consumption and provide adequate safety data, including acute and chronic toxicity, bacterial translocation, and sensitivity to vulnerable populations (Tompkins et al. 2008). In addition, some probiotic strains have potential risk of translocation and sepsis, which can pose serious health problems. Although there are a large number of potential probiotics, not all strains are identical. Since probiotics have many positive implications for human health, probiotic producers must choose the right strain and confirm that the source is safe, to avoid losing consumer trust or triggering safety incidents.

The Possibility of Horizontal Transfer of Antibiotic Resistance from Lactic Acid Bacteria

The researchers questioned the transfer of *tetW* from *B. animalis* subsp. *lactis* to other bacteria, including pathogens. Kazimierczak et al. have shown that the probability of gene-level metastasis in *B. longum* and *B. adolescentis* is quite low (Kazimierczak et al. 2006). At present, no studies have reported the transfer of *tetW* from *B. animalis* subsp. *lactis* to other bacteria.

In general, horizontal gene transfer occurs by transduction, transduction, transposon, or natural transformation (Levy and Marshall 2004). Transduction is mediated by phage, but few studies have been conducted on phage or bifidobacteria transduction. Transfection is often occurring in our intestines, but in many cases, the plasmid needs to transmit genes to the recipient cells. Since most *tetW* genes are located on ribosomal DNA rather than in plasmids, it is unlikely that *tetW* will be transferred by trans-binding (Ammor et al. 2008a; Ammor et al. 2008b).

It has been reported that transposons such as Tn916 and Tn5276 are mediated by the horizontal transfer of genes in lactic acid bacteria (Nawaz et al. 2011; Clementi and Aquilanti 2011; Mathur and Singh 2005). Although a recent research suggested that the transposable gene *tetW* of the *B. animalis* subsp. *lactis* is upregulated and the co-transcription of the *tetW* gene is identical to the transposase gene, there is no evidence that *tetW* participates in transposon expression (Gueimonde et al. 2010). In addition, since the recipient cells can accept naked strands of DNA, natural transformation allows all chromosomal sequences to be transferred to the recipient cells in a condition acceptable to the recipient cells. Although natural transformation can only change a few restricted species, studies have reported surprising results that *Vibrio cholerae* has never displayed the natural transformation of antibiotic resistance genes (Yamamoto et al. 2010; Meibom et al. 2005). In their report, antibiotic resistance genes can be transferred to *Vibrio cholerae* by natural transformation only when the growth conditions are very similar to the natural environment, such as on the surface of the crab shell; antibiotic resistance genes can be transferred to *Vibrio cholerae* by natural transformation. However, even the same strain did not undergo natural transformation in the same environment in vitro. It can be speculated that natural growth conditions include the presence of some key substances (in this case chitin), high cell density, auxotrophy, or stress conditions, in which case competent cells are produced and natural transformation is activated.

Considering that the cell density in our intestinal environment is much higher than that in laboratory using in vitro liquid culture and the large strands of DNA released in dead bacterial cells are present around the intestinal bacterial cells, nutrient limitation in the intestine may be a normal state. Because the most readily available nutrients have been absorbed by humans, only the remaining nutrients are being contested by a considerable number of gut microbes.

In addition, it has been reported that bile salts normally present in the intestine can induce tetracycline-resistant phenotypic expression in bifidobacteria (Noriega et al. 2005). Therefore, the intestinal environment may be beneficial for natural

transformation. In the decades of research on streptococci, we have known that in the laboratory environment, only some streptococci have the ability to naturally transform (Tomasz and Hotchkiss 1964). However, a recent discovery of a novel mechanism for regulating natural transformation in *Streptococcus* suggests that this property may spread more widely in *Streptococcus* than in previous views (Havarstein 2010; Johnsborg and Havarstein 2009). Moreover, Chen and Dubnau pointed out that various bacteria are competent and have their own DNA extraction system, which indicates that a variety of bacteria in the human gut can occupy naked-stranded DNA (including *tetW* and flanking sequences), although further discussion and investigation can prove that the *B. animalis* subsp. *lactis* may be the origin of the widely spread *tetW* gene in the food chain, but this possibility cannot be denied (Chen and Dubnau 2004).

Antibiotic Resistance Gene in Lactic Acid Bacteria

To the best of our knowledge, lactic acid bacteria such as *L. crispatus*, *L. plantarum*, *L. johnsonii*, *L. reuteri*, and *E. faecalis* are antibiotic resistant, containing antibiotic resistance genes ermB, tetM, and tetW (Klare et al. 2007; Mathur and Singh 2005).

Since most of these lactic acid bacteria are used as starters for meat and dairy products or as probiotic products, they may present a potential risk of transferring these genes to intestinal bacteria and pathogenic bacteria. In particular, some *Lactobacillus* and *Enterococcus* contain plasmids, and in vivo studies have shown that plasmid-carrying antibiotic resistance genes in *Lactobacillus* can be transferred to other bacteria (Jacobsen et al. 2007). Since many bacteria contained in the human intestinal flora can obtain exogenous DNA through conjugation, regular horizontal gene transfer among bacteria can occur in our intestines.

In view of the above problems and based on the fact that bacteria isolated from dairy products and meat products carry antibiotic resistance genes, bacterial strains carrying these resistance genes should not be used as feed additives, unless it can be proved to be caused by chromosomal mutations. That is recommended by the European Food Safety Authority (EFSA) technical manual for animal feed additives and products in the FEEDAP section.

Evaluation of Drug Resistance

With the increasing use of antibiotics and the application of some new antimicrobial compounds to treatment, drug resistance appears in the treatment, and with the spread of bacteria, the food chain is also one of the ways in which drug resistance between humans and animals spreads. Concisely, cured meat products and fermented dairy products are a major mediator of bacterial resistance (Versporten et al. 2016; Ponce de Leon-Rosales et al. 2015; Coccolini et al. 2015). Therefore, it is important to conduct anti-antibiotic gene detection and its potential transmission

mechanism for lactic acid bacteria used in foods, which can effectively reduce the risk of lactic acid strains carrying drug-resistant genes infecting hosts (Ramakrishnan and Sriram 2015; Koga et al. 2015).

Zhang et al. performed a comprehensive safety assessment based on genome-wide sequence and corresponding phenotype of *Lactobacillus plantarum* JDM1, a commercial probiotic strain widely used in China (Zhang et al. 2012). At the same time, the minimum inhibitory concentration (MIC) of JDM1 against 16 antibiotics and the production of biogenic amines by traditional oral toxicity test were determined. Overall, JDM1 contains 51 antibiotic resistance-related genes, 126 virulence-related genes, and 23 adverse metabolic-related genes. However, this strain does not contain a gene encoded by toxin or hemolysin, and the safety-related gene can hardly be transferred. This approach can be extended to provide a deep safety survey of new probiotic strains and greatly reveal its underlying risk factors and molecular mechanisms. However, not all genes are effective depending on environmental conditions, so this analysis only reveals the theoretical maximum level of risk.

1. *Antibiotic Sensitivity Test*

In the safety evaluation of lactic acid bacteria, the evaluation of drug resistance is very important. Twenty-four antibiotic resistance tests were carried out on 22 strains of *Lactobacillus* and 9 strains of *Bifidobacteria*, which are commonly used in Chinese probiotics and health-care products. The results showed that *Lactobacillus* was higher than the *Bifidobacteria* in the resistance to the antibiotics selected for the experiment. And all strains are more resistant to nalidixic acid, vancomycin, and fosfomycin.

2. *Antibiotic Resistance Gene Detection*

Numerous studies have confirmed that almost all antibiotic resistance in lactic acid bacteria is nonmetastatic, but drug resistance factors associated with binding plasmids and transposons may cause drug resistance to shift among intestinal flora (Fruchart et al. 1997).

11.2.1.3 Evaluation of Harmful Metabolites of Lactic Acid Bacteria

The detection of D-lactic acid is classified into L-lactic acid and D-lactic acid according to the difference in optical activity. Since the human body only contains enzymes that metabolize L-lactic acid, excessive D-lactic acid may cause metabolic disorders and even acidosis. The normal body does not cause obvious disease response to a small amount of D-lactic acid, but it can cause D-lactic acidosis, intestinal discomfort, and even coma for patients with short bowel syndrome. It is unsuitable for infants and other susceptible people to use D-lactic acid-producing lactic acid bacteria (Fukushima et al. 2014).

The nitroreductase activity was tested to demonstrate that the lactic acid bacteria have strong nitrate reductase activity. When the human body ingests foods contain-

ing more nitrate components, it will be reduced to nitrite by the bacteria in the body and then formed carcinogen nitrosamines or other intestinal endotoxins. Marino et al. tested their amino decarboxylase activity against lactic acid bacteria isolated from Italian cheese according to the method of Bover-Cid and Holzapfel (Marino et al. 2008).

In addition, due to the presence of amino acid decarboxylase activity in some lactobacilli, decarboxylation occurs in foods to reduce the amino acids to biogenic amines (Bover-Cid and Holzapfel 1999; De Llano and Cuesta 1998). The substances gradually accumulate in the human body, and excessive amounts lead to poisoning. At the same time, the decarboxylation reaction also leads to the production of nitrosamines. Therefore, before the products enter the market, the activity of amino decarboxylase should be tested on related lactic acid bacteria products. The method can refer to Marino et al. detecting the amino decarboxylase activity of lactic acid bacteria in Italian cheese (Marino et al. 2008).

11.2.2 Safety Evaluation Specifications for Different Functional Lactic Acid Bacteria Products

11.2.2.1 Safety Evaluation Specifications for Lactic Acid Bacteria in Food

Safety evaluation is the most basic requirement for functional lactic acid bacteria. Although most of the functional lactic acid bacteria are currently isolated from traditional fermented foods or human intestinal microbial systems, we cannot fully guarantee their safety as their particularity as food. In previous reports, it was also found that some lactic acid bacteria were isolated in the diseased or infected tissues. In some cases, specific lactic acid bacteria also turned into conditional pathogenic bacteria. It can be seen that the safety evaluation of lactic acid bacteria in functional probiotic foods is particularly important.

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have proposed guidelines for the safety evaluation of probiotic strains in foods. The necessary evaluation steps include drug resistance evaluation, human intervention evaluation, metabolic activity evaluation, epidemiological evaluation in circulation, animal-targeted infection evaluation, and special activity evaluation, such as hemolysis test evaluation, toxigenic test evaluation, and other basic aspects. Classifying from the experimental categories, the safety evaluation of lactic acid bacteria is generally divided into pathogenicity, toxicity detection, metabolic activity detection, and strain intrinsic property detection. Classifying from the subject, it is generally divided into in vitro experiments, animal targeting experiments, and human clinical intervention experiment.

Many studies report the health benefits of probiotics, such as improving the intestinal environment, enhancing immunity, reducing the risk of allergies, reducing diarrhea, improving constipation, and reducing the risk of cancer (Xie et al. 2014; Farid et al. 2011; Preter et al. 2007; Fujii et al. 2006; Xiao et al. 2006; Ogata et al.

1999; Colombel et al. 1987). Consumers purchase probiotic products based on these expectations. However, some of the concerns about the safety of probiotics, including the infection (such as sepsis) and antibiotic resistance genes, have been reported (Whelan 2010; Courvalin 2006; Snydman 2008). Since most probiotic consumption is food rather than drug, any potential risk factors for human health within the probiotic product must be excluded.

Another unique feature is that probiotics can be ingested by a variety of people at any time, including susceptible people, such as infants, children, the elderly, and patients. Therefore, not only the effectiveness to healthy people needs to be considered but also the safety of risk groups (Liong 2008). In 2008, an important issue concerning the safety of probiotics was reported. Clinical studies of severe acute pancreatitis in the Netherlands have shown an increased risk of mortality by probiotics (Besselink et al. 2008a). This finding warns of social and scientific research that the safety of probiotic must be taken seriously and considered.

Probiotic products have been widely used in foods in the world, such as yogurt, baby formula, and dietary supplements. Some risk factors such as drug resistance are present in probiotics and can be spread globally. In addition, many antibiotic resistance genes are present in intestinal bacteria has been reported. We need to continue to pay attention to these concerns. At the same time, the quality of probiotic products is another important part of the safety of probiotics. Probiotics have been added to many infant or child formulas in recent years (Abe et al. 2009). Since pathogenic bacteria such as *Salmonella* contamination is one of the biggest problems with formula, the production of probiotic powder in formula requires high hygienic specifications (Cahill et al. 2008). Besides, allergen contamination can cause serious problems; in order to make consumers consume probiotic products safely, these potential problems should be solved. .

In recent years, people have gradually begun to understand the benefits of LAB to the human body. Toxic diarrhea, antibiotic-induced diarrhea (AAD), allergies, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and constipation can be improved by probiotics. The different strains have different degrees of beneficial effects on acid and bile salt tolerance, intestinal adhesion, clinical-specific effects, and host health. LAB are one of the most widely used probiotics. It is generally accepted that the use of LAB to ferment products is feasible and safe. However, a few studies have shown that LAB can be isolated from patients with endocarditis, sepsis, liver abscess, and urinary tract infection, so the safety of LAB has attracted people's attention. Lara-Villoslada et al. (2007a, b) evaluated the oral toxicity of the isolated probiotic *L. coryniformis* CECT5711 and *Lactobacillus gasseri* CECT 5714 (Lara-Villoslada et al. 2007a). In an oral toxicity analysis of 20 Balb/c mice after oral administration of CECT5711 or CECT5714 (10^{10} cfu/d) for 30 days, the results indicated that CECT5711 and CECT5714 do not have harmful enzyme activity and inherent antibiotic resistance characteristics. After ingestion of the two strains, the mice had no side effects on their body weight or diet, no bacteremia in the liver or spleen, and no treatment-related bacterial translocations in these organs. The liver glutathione content and plasma lipid peroxidation concentration of mice in the probiotic group were not statistically different from the control group.

Probiotic treatment did not cause changes in biochemical and blood parameters, so it can be inferred that CECT5711 and CECT5714 are not pathogenic and may be safe for human health. It is necessary to evaluate the safety of newly selected LAB that can be used as probiotics, especially for food.

At present, the safety evaluation of LAB in domestic and abroad is mainly aimed at the toxicological evaluation of foods with microorganisms as raw materials or starter. It is necessary to conduct toxicity tests, including three teratogenic mutation experiments and 30d feeding experiments.

Yakabe et al. (2009) reported that the probiotic *Lactobacillus* sp. KB290, isolated from plant, has functions to improve intestinal health and stimulate immunity (Yakabe et al. 2009). The genotype; acute, subacute, and subchronic toxicity; and the potential for bacterial migration of KB290 were studied deeply. The result of bacterial back mutation experiments, using preincubation method, showed that KB290 was not mutagenizable. In a single oral toxicity test, the maximum nontoxic amount of KB290 was 10^9 cfu/ml (20 ml/kg). In a subacute trial, after daily administration of 10^8 cfu/kg, 10^9 cfu/kg, or 10^{10} cfu/kg of KB290 to rats for 2 weeks, we did not observe a significant therapeutic-related effect or the evidence of bacterial translocation in the gastrointestinal tract. When the rats were orally administered with KB290 for 13 weeks (subchronic test), there was still no significant treatment-related effect and no significant toxicological effects. Based on these results, we believed that the highest dose tested (10^{10} cfu/kg/d) is the level of no visible harmful effects (NOAEL). These results indicated that KB290 is safe for human.

1. Salmonella Reverse Mutation Assay

The Salmonella Reversal Mutation Test (Ames Test) was established and developed by Ames and his team at the University of California for more than 10 years. This method has been widely used for the detection of mutations and antimutagenic ability and the screening of carcinogens because of its advantages of speediness, simplicity, sensitivity, economy, and suitability for testing mixtures. Ebringer et al. (1995) tested the mutation and anti-mutation ability of *Lactobacillus* spp. CCM4179 and CCM4180 by Ames test (Ebringer et al. 1995). The results showed that there was no obvious mutagenic effect.

2. Bone Marrow Micronucleus Test

This method is suitable for determining the mutagenic effects of a substance. In the late stage of cell mitosis, when chromosomes enter a daughter cell to form a nucleus, the chromosome or chromatid fragment remaining in the cytoplasm is called a micronucleus. After the terminal phase, a subnucleus that is much smaller than the nucleus is formed. Domestic researchers performed bone marrow micronucleus experiments on Yiliduo brand active LAB milk drink using SPF-class NIH mice. The micronucleus test results were negative at doses of 5.00–40.00 g/(kg·BW), indicating that Yiliduo had no mutagenic effects at the somatic level.

3. Mice Sperm Abnormality Test

Many chromosomal genes directly or indirectly determine the morphology of sperm, and sperm abnormalities are the result of genetic mutations, indicating changes in related genes and their protein products. The method is suitable for evaluating the genotoxicity of health foods to male germ cells.

The detection of total glutathione in the liver. The reduced glutathione (GSH) exerts the ability to resist oxidation and scavenge free radicals mainly through the active sulfhydryl group on the side chain of cysteine and clinically acts as an important pharmaceutical ingredient for protecting liver. Lara-Villoslada et al. (2009) studied the GSH level in liver tissue after continuously giving *L. fermentum* CECT5716 to Balb/c mice for 2 days (Lara-Villoslada et al. 2009). The GSH levels before or after gavage were basically equal, indicating that the CECT5716 had no negative effects on liver function. In addition, it has been reported that *L. rhamnosus* CCFM1107 can improve alcoholic liver injury (Tian et al. 2015).

The detection of serum malondialdehyde (MDA) concentration. Peroxidation reaction occurs with the free radicals in the organism acting on the lipids. The final product is malondialdehyde, which causes cytotoxicity as the polymerization of molecules, such as proteins and nucleic acids. Due to the low concentration and instability of free radicals in the body, the MDA level is generally used for reflecting the metabolism of free radicals in the body. Therefore, it can indirectly reflect the damage degree of cells attacked by free radicals. Lara-Villoslada et al. (2009) used HPLC to determine the MDA level in the blood after continuous gavage of *L. fermentum* CB5716 for 28 days in Balb/c mice (Lara-Villoslada et al. 2009). The results showed that there was no significant difference between the mice in CB5716 group and control group.

The detection of serum amyloid A (SAA) protein. SAA, an acute phase-reactive protein, is associated with high-density lipoprotein (HDL) and can regulate HDL metabolism during inflammation. SAA concentration is a sensitive indicator of inflammation in the early stages of infectious diseases and a plasma indicator for the analysis of sepsis. Lara-Villoslada et al. (2007a, b) intraperitoneally injected *L. salivarius* CECT5713 in Balb/c mice; SAA level in blood was detected by ELISA (Lara-Villoslada et al. 2007b). The results showed that SAA concentration increased in the short term after injection but can return to normal levels soon.

The detection of bile salt hydroxylase activity. Some LAB have the ability to degrade bile salts (Ren et al. 2011). If bile salt dissociates in the small intestine, it will affect the digestion and absorption of fat. If it does not dissociate in the large intestine, it also will affect the enterohepatic circulation and then affect the digestion and absorption of fat. Bacterial metabolism affects the content of secondary bile salts in bile acids, which can directly affect the activity of bile salt hydroxylase and indirectly affect the solubility of bile salts by affecting the acidity and alkalinity of the colon. Therefore, LAB do not have bile salt hydroxylase activity in vivo. Some studies examined the concentration of bile salt hydroxylase in LAB by MRS culture in vitro and isotope tracing methods in vivo (Wang et al. 2015; Abouakil et al. 1989).

11.2.2.2 LAB Evaluation Criteria in Clinical or Health Care

The Joint Working Group of FAO and WHO drafted new guidelines for the probiotic evaluation in food in May 2002. FAO and WHO and the countries they represent have established evaluation criteria based on the definition of probiotics and the minimum requirements for accurate health verification. Although the FAO and WHO reports were primarily in food field, a number of recommendations including the definition of probiotics were adopted at the International Science Conference in May 2002. Based on these guidelines, several important specifications and standards must be introduced to ensure that physicians know the quality and reliability of the products they prescribed or recommend, the primarily points as shown in the following.

Strain Identification

The first consideration is using internationally recognized methods to confirm and represent the genus and species of strain, such as DND-DNA hybridization and 16S rRNA-encoded DNA sequencing (Wang et al. 2002). The most important methods of strains differentiation are pulsed electric field gel electrophoresis and random amplified polymorphic DNA technology. Strain differentiation and characterization can also be performed by determining the genetic elements other than chromosomes, such as plasmids. Once the strain has been identified, it must be named according to bacterial name approval list and update the list.

The second consideration is to define a strain name according to the probiotic function that is consistent with its characteristics, such as *L. rhamnosus* GG (LGG). This will enable doctors and consumers to track publications related to the strain and verify the strain do exhibit the probiotic properties. An important example of a strain name causing confusion is that a so-called probiotic yogurt mentioned in a publication has side effects on patients, but in fact, 12 of the 16 strains mentioned have no probiotic characteristics (Sipsas et al. 2002). Similarly, the benefits of strains claimed on a website or label, which are not in the product or never been proven (Reid et al. 2001). This is not to say that such products are defective or unreliable, but manufacturers need to improve the probiotic characteristics of the products.

In Vitro and In Vivo Tests

In the early 1980s, some research teams discovered probiotic strains of *Lactobacillus* and provided a useful screening system in vitro. Therefore, characteristics such as cell adhesion, acid production, bacteriocin, hydroperoxide, and ability to inhibit adhesion of pathogens are considered to be the most important factors for conferring functional properties of probiotics (Gopal et al. 2001; Reid and Bruce 2001). These methods can also be used for strain differentiation, but a single method is not

sufficient. In short, before these factors are used for predicting the effects of probiotics on the human body, they need to be expressed in vivo and verify the main mechanism. In vitro tests such as bile salt tolerance can be associated with gastric survival tests in vivo; spermicide resistance can help probiotics survive in the vagina.

Many other trials that need to be validated in vivo still have advantages in assessing and characterizing the body and studying the underlying mechanisms. For example, *Lactobacillus* can adhere to intestinal cells and signal mucus products, thereby preventing the adhesion of pathogens, which was very valuable and proposed a new function of probiotics in the intestine. In addition, the study also revealed the mechanism by which *Lactobacillus* interferes with the pathogenesis of *E. coli*, including analysis of the inflammatory effects of lipopolysaccharide antagonized by competition of lipoic acid with soluble CD14 (lipopolysaccharide binding protein) (Vidal et al. 2002). The important concept shows that probiotics can directly benefit the host.

The Safety

Probiotics are living organisms, so they can infect the host. Historical data indicate that LAB and bifidobacteria in the form of capsules are safe for humans. This conclusion is supported by their normal symbiosis with bacteria in mammals and their safe use in different food and supplement products. Despite this, it has been reported that they have side effects, including rare systemic infections. Some susceptible populations, such as immunocompromised individuals and those with symptoms of intestinal bleeding, should be cautious when taking live bacteria (Marteau 2002). Patients with susceptibility to arthritis or other complications should also be careful to ensure that they do not induce excessive immune stimuli.

In order to establish safety regulations for probiotics, FAO and WHO recommend the east tested groups of probiotic strains for experimental evaluation, including antibiotic resistance evaluation, metabolite activity, toxin production, hemolytic activity, infection in immunocompromised animal models, and incidence of side effects in the human and in consumers. But the side effects of *Lactobacillus* strains are very few. A large number of studies confirm their functional effects, and few studies research side effects.

Phase II of Clinical Study

The second phase of the clinical study primarily assessed the effectiveness of a product relative to placebo. For an individual, clinical outcomes should have statistically and biologically significant improvements in symptoms, life quality, reduction in disease risk or duration, and rapid recovery from disease. More credible clinical evidence is needed in a wider range of clinical examples. These studies need to provide the doctor with the strain name, form of production, and the effectiveness of these strains in some respects. *L. rhamnosus* GG and *L. reuteri* SD2222 have a lot

of good clinical data, but it is not possible to simply apply the strains to the clinic. The LGG strain is a liquid form product from Finland, while the US product is put into a capsule. The study of SD2222 is even less clear with fewer articles in the relevant reports, and it is not a product that can be sold in labels in the United States.

Phase III of Clinical Study

The third phase of the clinical study is to evaluate the effectiveness of the product compared to the standard treatment of a particular disease. In general, on the one hand, it is necessary to provide the results of randomized and double-blind experiments, determine the sample amount and use it reasonably, and obtain truthful results. Such experiments need to consider life quality assessments and risk-benefit ratios. For example, 3-hydroxy-3-methylglutaryl coenzyme as a reductase inhibitor product may reduce HDL cholesterol levels by 45% but may also cause rhabdomyolysis, kidney damage, or death. On the other hand, the results of animal experiments showed that taking 10^4 cfu/d of *L. reuteri* CRL1098 for 7 days can prevent hypercholesterolemia and have a 17% probability of causing HDL to become LDL (Taranto et al. 2000). It may be reappeared in the human body and then will provide a significant improvement in clinical treatment to no side effects. In other words, careful planning and extensive evaluation for results are required before deciding whether probiotics should be applied to phase III clinical trials.

Health Claim and Product Launch

In more cases, probiotics are allowed in foods only in general health conditions. In terms of drug use, it is demonstrated that probiotics were superior to placebo in certain situations. A three-stage clinical study is required for a specific condition at the time of drug review. Clear and specific statements and labels should be marked on the market to let patients know the true benefits of this probiotic drug. For example, statements such as the information of "reducing the incidence and severity of infant rotavirus diarrhea" are more accurate than "improving gut health." In addition, the disclaimers stated on some company websites are enforced unless they can be proven, which will greatly increase consumer trust for product (Reid et al. 2001).

11.2.2.3 Criterion of LAB for Feed and Crops

The safety of microorganisms as feed additives needs to be standardized and improved. In addition to the existing additives and veterinary drug management regulations, some factors, such as animal safety, consumer safety, and environmental safety, must be considered. In the application process, it should be proved that it is safe and harmless through rigorous experiments before it can be widely applied.

The Identification of LAB

Since LAB are generally recognized as safe (GRAS), the strains added to the feed must belong to the certification safety catalog, and strain identification is the basis of safety regulations. Bacterial identification can be performed by 16S rDNA sequence analysis, PCR product analysis, and so on. Berger's *Manual of Systematic Archaea and Bacteriology* (2015) can be used for reference if necessary, including strain isolation and purification, morphological observation, sugar fermentation experiments, and physiological and biochemical property analysis.

Operating Environment and Personnel Safety

In the process of feed production and addition, it is necessary to ensure the safety of animals and breeders and keep qualified environment. Therefore, it is significant to pass the drug resistance and toxicological tests of LAB, including general drug resistance test- and toxicology test-related experiments, such as carcinogenicity test, acute toxicity testing, and so on. At the same time, considering the relative balance of the types and quantities of microorganisms in the environment and the self-regulation within a certain range, large-scale feeding of single or multiple LAB strains will impact the number of bacteria in the surrounding environment and will endanger the microbial ecosystem once the formation of dominant flora. This hazard will not have a major impact in the short term, but it will also significantly change the composition of environmental microbes in the long-term, posing a potential hazard on maintenance of ecological balance.

Safety for Animals

Although the breeding cycle is relatively short from the birth of the animal to the slaughter, the long-term LAB feeding experiments are still necessary, which can most directly prove the harmlessness of the product. Generally, long-term feeding experiments need to be fed for 5–7 years, which proves that it has no adverse effects on animal growth and development. In addition, the effects of feeding LAB feed on animal reproduction must be further verified. When its safety on heredity has been verified, the product's safety can be determined, and thus it can be widely launched.

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