Probiotics and safety[1–3]

Norio Ishibashi and Shoji Yamazaki

ABSTRACT Bacterial species that have traditionally been regarded as safe are used in probiotics; the main strains used include lactic acid bacteria and bifidobacteria that inhabit the intestinal tracts of humans and animals. However, reports of frequent isolation of bacteria used in probiotics from infection sources in recent years have raised much debate over the safety of probiotics. This article describes the status quo of isolation of probiotic bacteria from infections and reviews each of the factors that have to be addressed in assessing the safety of probiotics, namely pathogenicity, infectivity, toxicity, and intrinsic properties of the bacteria. Monoassociation with *Bifidobacterium longum* in gnotobiotic mice as a method to assess safety with respect to infection, and translocation and immune responses as a result of the monoassociation are also described. *Am J Clin Nutr* 2001;73(suppl):465S–70S.

KEY WORDS Probiotics, safety, lactic acid bacteria, bifidobacteria, infection, translocation, immunity

PROBIOTIC BACTERIA

Probiotics can be defined as a food (feed) or drug containing live microbes that, when ingested, is expected to confer beneficial physiologic effects to the host animal through microbial actions (1). Microbial components and metabolites are essentially excluded from the definition of probiotics. Microbes used in probiotics should be able to express their activities in the host body. The first consideration is the bacteria that normally inhabit the intestinal tract, and ingestion of these bacteria may affect the intestinal microbial balance. The human digestive tract is inhabited by numerous microbes (2). The balance of this microbial flora greatly influences the intestinal environment (3). Among the numerous intestinal microbes, those that are expected to beneficially affect the host by improving the intestinal microbial balance, and hence are selected as probiotics, include species of the genera *Lactobacillus, Bifidobacterium*, and *Enterococcus* (4, 5). The representative species include *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Enterococcus faecalis*, and *Enterocuccus faecium*. *Bifidobacterium* species that specifically inhabit the intestinal tracts of animals, such as *Bifidobacterium thermophilum* and *Bifidobacterium pseudolongum*, are used in animal probiotics (6). Some bacteria that do not normally inhabit the intestinal tract may also come under the category of probiotics. They are used as starters in dairy products and include mainly *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Leuconostoc* and *Lactococcus* species. However, these bacteria do not colonize the intestinal tract and their effect on intestinal microbial balance is expected to be small (7).

SAFETY OF PROBIOTICS

Most probiotics are marketed as foodstuffs or drugs. Consideration of the safety of probiotics is therefore of utmost importance. The safety of the microbes that have been used traditionally in probiotics has been confirmed through a long period of experience. Bacteria such as *Lactobacillus*, *Leuconostoc*, and *Pediococcus* species have been used extensively in food processing throughout human history, and ingestion of foods containing live bacteria, dead bacteria, and metabolites of these microorganisms has taken place for a long time (8, 9). Ecologically, bifidobacteria are present as the predominant bacteria in the intestinal tract of breast-fed infants and are considered to contribute to the health of infants (3, 10). Until now, the safety of these microbes has not been questioned, and reports of a harmful effect of these microbes to the host are rare. However, in recent years, many species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* were isolated frequently from various types of infective lesions. According to Gasser (11), *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. casei*, *Lactobacillus paracasei*, *Lactobacillus salivarius*, *Lactobacillus lactis*, and *Leuconostoc mesenteroides* are some examples of lactobacilli isolated from bacterial endocarditis; *L. rhamnosus*, *L. plantarum*, *Leuc. mesenteroides*, *Pediococcus acidilactici*, *Bifidobacterium eriksonii*, and *Bifidobacterium adolescentis* have been isolated from bloodstream infections and many have been isolated from local infections. Gasser excluded *Enterococcus* and *Streptococcus* from his review because both contain frankly pathogenic species, and *Bifidobacterium eriksonii* was recently reclassified as *Bifidobacterium dentium* (12). Because *B. adolescentis* and *B. dentium* have similar phenotypic characteristics, such as carbohydrate fermentation activities, *B. adolescentis* isolated from infections may

[1] From the Nutritional Science Laboratory, Morinaga Milk Industry Co Ltd, and The Public Health Institute, Tokyo.

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[3] Address reprint requests to N Ishibashi, Nutritional Science Laboratory, Morinaga Milk Industry Co Ltd, 1-83, 5-chome, Higashihara, Zama-shi, Kanagawa-pre, 228-8583, Japan. E-mail: n_ishibs@morinagamilk.co.jp.

TABLE 1
Lactic acid bacteria and bifidobacteria isolated from endocarditis, bacteremia, and bloodstream and local infections[1]

Genus	Species
Lactobacillus	*Rhamnosus, plantarum, casei, paracasei, salivarius, acidophilus, plantarum, gasseri, leichmanii, jensenii, confusus, brevis, bulgaricus, lactis, fermentum, minutus,* and *catenaforme* sp.
Lactococcus	*Lactis*
Leuconostoc	*Mesenteroides, paramesenteroides, citreum, pseudomesenteroides,* and *lactis* sp.
Pediococcus	*Acidilactici* and *pentosaceus*
Bifidobacterium	*Dentium (eriksonii)* and *adolescentis* sp.
Enterococcus	*Faecalis, faecium, avium*, and others

[1] From references 11, 14–17.

be classified as *B. dentium* by using genetic classification techniques (13). Aguirre and Collins (14) similarly reported the isolation of *Lactobacillus*, *Pediococcus*, *Enterococcus*, and *Lactococcus* species from infection sites. Brook (15) reported the isolation of *Bifidobacterium (dentium* and *adolescentis)* and some species of *Lactobacillus* from pediatric infection sources. Furthermore, Maskell and Pead (16) reported an increasing incidence of isolation of lactobacilli from patients in England and Wales and the detection of ofloxacin resistance in theses isolates. In addition, Jett et al (17) reported several virulence factors of enterococci. The species isolated from various infections are shown in **Table 1**. These reports raised much debate in recent years over the safety of probiotics. Adams and Marteau (18) reported the discussion in a European Union workshop about the safety of lactic acid bacteria by reviewing the published reports. The workshop concluded that, with the exception of enterococci, the overall risk of lactic acid bacteria infection is very low. However, it was decided that *L. rhamnosus* still warranted surveillance. Considering the fact that many of the bacterial species that constitute probiotics have actually been isolated from infection sites, verification of the safety of probiotics used industrially and commercially is important.

THE SAFETY OF PROBIOTICS

The factors that must be addressed in the evaluation of safety of probiotics include pathogenicity, infectivity, and virulence factors comprising toxicity, metabolic activity, and the intrinsic properties of the microbes. Donohue and Salminen (19) provided some methods for assessing the safety of lactic acid bacteria through the use of in vitro studies, animal studies, and human clinical studies and indicated that some current probiotic strains are reported to fulfill the required safety standards. Salminen and Marteau (20) also proposed studies on intrinsic properties, pharmacokinetics, and interactions between the host and probiotics as means to assess the safety of probiotics.

Pathogenicity and infectivity

The absence of pathogenicity and infectivity is a requisite of probiotic safety. The frequent isolation of lactic acid bacteria from clinical infections in recent years has raised debate over the safety of these bacteria and whether the bacteria are actually infective (18–21). However, even these lactic acid bacteria and bifidobacteria, long considered to have no infectivity, are isolated from infections; it is unlikely that they universally possess generalized infectivity. The isolation of lactic acid bacteria and bifidobacteria from infections is likely to be the result of opportunistic infections. The increasing isolation from clinical infections in recent years may be due to an increased awareness of the role of these bacteria in causing opportunistic infection. Although lactic acid bacteria or bifidobacteria may invade the host body by bacterial translocation or other routes, causing bacteremia (22, 23), for these bacteria to actually cause systemic infections, from endocarditis and other infections to septicemia, both the bacterial factors and the host factors probably need to be involved. However, the assessment of such an interaction is difficult. The safety of a bacterial strain may be evaluated by considering questions such as whether invasion of the host by the bacteria leads to infection, whether infection results in severe outcome, and the effect of association of the bacteria on the host.

Whether the probiotic bacteria are infective is difficult to prove, especially in anaerobes, which are generally considered to have no infectivity. Even if the bacteria are administered orally, infection does not normally occur in healthy animals; this is particularly so for bacteria with weak infectivity. Even with strongly infective bacteria, it is not easy to establish infection by using a single species, and various techniques are necessary to establish infection, such as the use of various pretreatments in the experimental system or the use of mixed infection (24). A bacterial single-administration (acute) toxicity test and a repeated-administration (chronic) toxicity test will provide some information on toxicity. For *B. longum* BB536, the median lethal dose (LD_{50}) obtained in single-administration toxicity tests in mice is >50 g/kg (5×10^{13}/kg), which was the technical maximum dose for oral administration, and 5×10^{11}/kg for intraperitoneal administration (25). With repeat oral administration, toxicity was not shown, even after a dose of 2.5×10^{11} kg/d was administered for 1 y. For *L. rhamnosus*, the LD_{50} with intraperitoneal administration was reported to be $1.7–3.6 \times 10^{9}$/mouse (26). Donohue et al (19, 27) summarized the results of various reports on acute toxicity tests of several strains of *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*. The data in these reports are fragmented and the studies were not conducted under the same experimental conditions, making direct comparison difficult.

As already mentioned, the isolation of lactic acid bacteria and bifidobacteria from infections is the result of opportunistic infection. The causes of opportunistic infection include skin injury, chronic diseases, cancer, and drug-induced abnormality. Bacterial translocations induced by these and other factors are also considered to play an important role. Bacterial translocation is a phenomenon caused by a diminished intestinal barrier, resulting in the passage of bacteria (or bacterial components or products) across the mucous membrane and epithelium. The bacteria are then transported through the tunica propria to the mesenteric lymph nodes (MLN) and other organs (**Figure 1**). This results in bacteremia, which may progress to multiple organ failure and septicemia (22, 28, 29). Endogenous infection as a result of translocation of intestinal bacteria is one cause of opportunistic infection in immunocompromised hosts (30, 31). Many factors may promote bacterial translocation by intestinal bacteria, including intestinal mucosal injury, immunodeficiency in the host, and an abnormal intestinal bacterial flora (overgrowth of intestinal bacteria) (32, 33). The route of bacterial translocation is thought to be via the MLN or the portal vein, but observation of the translocation of intestinal bacteria usually begins in the

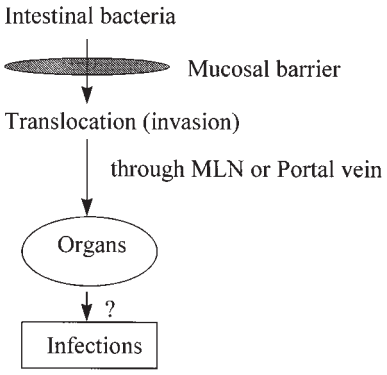

FIGURE 1. Proposed invasion route of intestinal bacteria by bacterial translocation (MLN = mesenteric lymph nodes).

MLN. Translocation from the intestine is difficult to induce in healthy animals (34). Therefore, artificial inducing techniques are used, such as antibiotic treatment, administration of an immnuosuppressive agent, or a combination of these (20, 35). Another method is to use germ-free animals. Although bacterial translocation does not occur commonly in healthy specific pathogen-free (SPF) animals, it is known to occur for a long duration in germ-free mice (36, 37). This phenomenon is caused by an immature intestinal barrier and an underdeveloped immunity of the lymphocytic system in germ-free animals. Berg and Garlington (36) observed translocation in *E. coli* or *L. acidophilus*-monoassociated gnotobiotic mice and found translocation to the MLN, spleen, and liver over a long duration. Maejima and Tajima (37) administered to germ-free mice a mixture of bacteria including *E. coli*, *Streptococcus faecalis*, and *Bacteroides* sp. isolated from conventional mice and observed translocation to various organs. Among the intestinal bacteria, *E. coli*, *Klebsiella* sp., and *Enterobacteriaceae* sp. translocate easily, followed by *Enterococcus*, *Staphylococcus*, and *Lactobacillus* species (K Itoh, unpublished observations, 1994).

Many probiotic bacteria inhabit the intestine and affect the intestinal ecology by competing with the intestinal flora. Interest has been shown in using the observations of translocation of a bacterial strain from the intestinal tract and the subsequent effects on the host as a method of evaluating bacterial infectivity or pathogenicity. Salminen and Marteau (20) proposed translocation and colonization properties for pharmacokinetics studies to assess the safety of probiotics. In the case of monoassociation of an antibiotic-resistant *E. coli* strain C25 to antibiotic decontaminated mice, translocation occurs and the systemic immunity is subsequently impaired (38). Feeding SPF mice by oral transparenteral nutrition induces bacterial translocation and leads to impairment of systemic immunity (39). In an extreme case in which pathogenic *E. coli* O111 or O157 is administered to germ-free animals, the bacteria proliferate in the intestinal tract, translocate, and cause death of the animal (40). In the case of *E. coli* O157, death is caused by nephritis (S Yamazaki, unpublished observations, 1998). Because the intestinal bacterial flora are known to affect the whole immune system of the host (41, 42), the effect of monoassociation of a bacterial strain in gnotobiotic animals and the effect of colonization and translocation on the immune system of the host have also attracted interest.

Yamazaki and others (40, 43–45) reported colonization, bacterial translocation, and immune responses in gnotobiotic mice monoassociated with *B. longum* BB536. When *B. longum* is administered orally to germ-free mice, the bacteria colonize the intestinal tract and reach a concentration of 10^9–10^{10}/g intestinal content in 2–3 d. Translocation of the colonized *B. longum* to the MLN, liver, and kidney occurs between 1 and 2 wk after the association, but the translocated *B. longum* causes neither infection nor any harmful effect. Furthermore, the translocated *B. longum* disappears after week 4, clearly showing inhibition of translocation (**Table 2**). The phenomenon of inhibition of translocation is not observed in nude mice and translocation persists without causing infection or any harmful effect. The inhibition of translocation observed in *B. longum*-monoassociated mice is thought to be associated with T-lymphocyte-mediated immunity. The time of occurrence of translocation inhibition coincides with the time of expression of cellular immunity in the *B. longum*-monoassociated mice (**Table 3**). *B. longum* monoassociation results in an increased production of total immunoglobulin A and anti-*B. longum* immunoglobulin A antibody (38).

TABLE 2
Translocation of *Bifidobacterium longum* into internal organs of germ-free athymic nude mice (*nu/nu*) of Balb/c background and *nu/+* littermates after *B. longum* monoassociation[1]

Mice	Wk after monoassociation	No. of mice	Cecal population/g	Isolation of *B. longum*		
				Liver	Mesenteric lymph nodes	Kidney
nu/+	1	3	10^9–10^{10}	2/3[2]	3/3	2/3
	2	3	10^9–10^{10}	2/3	3/3	3/3
	4	5	10^9–10^{10}	0/5	1/5	ND[3]
	8	5	10^9–10^{10}	1/5	1/5	1/5
	12	5	10^9–10^{10}	0/5	0/5	0/5
	18	5	10^9–10^{10}	0/5	0/5	0/5
nu/nu	1	3	10^9–10^{10}	2/3	3/3	3/3
	2	3	10^9–10^{10}	3/3	3/3	3/3
	4	4	10^9–10^{10}	3/4	4/4	4/4
	6	3	10^9–10^{10}	2/3	3/3	3/3
	12	2	10^9–10^{10}	2/2	2/2	ND

[1] From reference 45.
[2] No. positive/no. tested.
[3] Not determined.

TABLE 3
Immunologic responses in *Bifidobacterium longum* monoassociated mice to *B. longum* antigen[1]

	Time after monoassociation (wk)						
	1	2	4	6	8	12	18
Translocation of *B. longum*	+	+	−	−	−	−	−
Serum immunoglobulin G antibody	−	−	−	−	±	+	+
Immunoglobulin A antibody							
Serum	−	−	−	−	−	−	−
Bile	−	−	−	+	+	+	+
Cecal contents	−	−	−	−	+	+	+
Ileac wall	−	−	−	+	+	+	+
Cell-mediated immunity							
Footpad reaction	−	−	+	+	+	+	+
Macrophage migration inhibition	ND[2]	−	+	ND	ND	ND	ND

[1] From reference 45.
[2] Not determined.

Another interesting finding is that a lower toxicity is observed when *B. longum*-monoassociated mice are challenged with *E. coli* O111 or O157. When *E. coli* O111 or O157 was administered orally to germ-free mice, translocation to various organs occurred and the mice died by endotoxin shock or organ failure. In the case of *E. coli* O157, the mice developed nephritis and all died within 5 wk. When *B. longum*-monoassociated mice were challenged with *E. coli* O157, the intestinal count of *E. coli* O157 was suppressed at a low concentration and no death was observed in 5 wk. When *B. longum*-monoassociated mice were challenged with *E. coli* O111 at a lethal dose, death was avoided (**Table 4**). Furthermore, when *B. longum*-monoassociated mice were challenged with a sublethal dose of *E. coli* O111, translocation was observed in the beginning but became totally undetectable after 7 d. In contrast, translocation in germ-free mice was observed for >12 wk (*B. longum*-unassociated) (**Table 5**). Although the immune responses induced by *B. longum*-monoassociation in germ-free mice requires further analysis, the results of all the above studies suggest augmentation of the host immune functions by *B. longum* monoassociation.

The pathogenicity and infectivity of a bacterium cannot be determined solely by using a monoassociation model in germ-free mice and studying translocation and subsequent changes; nevertheless, these studies showed that monoassociation and translocation of *B. longum* BB536 do not produce infection or any harmful effect on the host but, conversely, augment the host immunity. These findings suggest that this experimental system may be useful as one method for evaluating the safety and usefulness of probiotics.

Metabolic activity (enzymatic activity associated with production of toxic substances)

Another requisite of probiotics is that the probiotic bacteria should not produce harmful substances by metabolic activities. One test is to determine whether the bacteria convert food components or biological secretions into secondary substances harmful to the host. For example, some intestinal bacteria act on proteins and their digested products to produce ammonia, indol, phenols, and amines (46). Although *Lactobacillus* and *Bifidobacterium* species have not been reported to produce very harmful compounds, the data on the production and consumption of ammonia are interesting. Araya-Kojima et al (47, 48) measured the enzyme activities related to the consumption and generation of ammonia in *Bifidobacterium* sp. of human origin. Compared with other bacteria of the intestinal flora, *Bifidobacterium* sp. have a lower deaminase activity involved in the production of ammonium from amino acids but a higher ammonia assimilation activity. Secondary bile acids are important harmful substances that are produced by intestinal bacterial actions on body secretions. They may exhibit carcinogenicity by acting on the mucous-secreting cells and promoting their proliferation, or they may act as promoters of carcinogenesis (49). Many intestinal bacteria, including *Bifidobacterium* and *Lactobacillus* species, can deconjugate conjugated bile acids (50). However, *Bifidobacterium* [5 species (51) and 10 species (52)], *Lactobacillus* (5 species), *Leuconostoc lactis* subsp. *lactis*, and *S. thermophilus* have been reported to lack the 7α-dehydroxylase activity that is related to the production of secondary bile acids (51, 52). For *Enterococcus*, cytolytic substance and other virulence factors were reported by Jett et al (17).

Platelet-aggregating activity, mucus degradation activity, and antibiotic resistance

Platelet aggregating activity has been considered to be a required test in the assessment of safety. Aggregation of platelets by bacteria is thought to contribute to the progression of infective endocarditis (53). Harry et al (54) measured the platelet-aggregating activity of strains of *L. rhamnosus* and *L. paracasei* subsp. *paracasei* isolated from infective endocarditis; laboratory strains of the same species; and *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus oris*, *L. plantarum*, and *L. salivarius*. The platelet-aggregating activity differs according to strains; 5 of 5 strains of *L. rhamnosus* were isolated from infective endocarditis, and 8 of 16 laboratory strains of *L. rhamnosus* showed aggregating activity. The aggregation is thought to be associated with the proteins on the outer cell layer. The

TABLE 4
Protective effect of *Bifidobacterium longum* monoassociation against lethal per os challenge with *Escherichia coli* O111[1]

	Mortality	
Type of mouse	6 h	18 h
B. longum[2]	0/10	0/10
Germ-free	0/11	7/11 (64%)[3]

[1] From reference 45. Dose of *E. coli*: 10^{10} viable units/mouse.
[2] *B. longum*-monoassociated mice.
[3] $P < 0.01$.

TABLE 5
Reduction of translocation of *Eschericia coli* O111 in germ-free and *Bifidobacterium longum*-monoassociated mice[1]

Organ	Time after *E. coli* association			
	1 d	3 d	7 d	14 d
Liver				
Germ-free mice	10^4–10^5 [2]	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice[3]	10^3–10^4	10^2–10^3	—	—
Spleen				
Germ-free mice	10^2–10^3	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice	10^3–10^4	10^2–10^3	—	—
Kidney				
Germ-free mice	10^4–10^5	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice	10^2–10^3	10^2–10^3	—	—
Lung				
Germ-free mice	10^3–10^4	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice	10^3–10^4	10^2–10^3	—	—

[1] From reference 45. Translocation in germ-free mice continued for >3 mo.
[2] Viable cell of *E. coli*/g organs.
[3] *B. longum*-monoassociated mice.

properties of the outer cell layer have been measured by hydrophobicity, hydroxyapatite adhesion, and salivary aggregation. *L. rhamnosus* strains isolated from infective endocarditis have higher activities than do laboratory strains of *L. rhamnosus* (55). As for the other virulence factors, the activities of glycosidases and proteases (arylamidase), which might enable the breakdown of human glycoproteins and the synthesis and lysis of human fibrin clots, have been measured in *L. rhamnosus*, *L. paracasei* subsp. *paracasei*, and other strains. Some strains produce these enzymes, suggesting that they may have an infective property in causing endocarditis (56). Ruseler et al (57) measured the enzymatic activities relating to degradation of intestinal mucus glycoprotein in several strains of *Lactobacillus* and *Bifidobacterium* and found no such activity in these strains. Further research on the structure of the outer cell layer or the above-mentioned enzyme activities in probiotic bacteria is expected. Whether the outer layer structure, which contains surface proteins, glycoproteins, and lectins, is really related to infectivity and whether glycosidases, proteases (arylamidase), and other enzymes capable of degrading human intestinal cells are related to infection remain to be elucidated. If these relations are proven, the implication for the assessment of human intestinal cell adhesion, which has been regarded as a necessary property of probiotics (58), requires further consideration.

The issue of the isolation of antibiotic-resistant bacteria has also been raised (16, 59). Especially in the case of *Enterococcus*, many strains, including those isolated from infection sites, have been shown to be multiply resistant to many antibiotics (60). These resistant bacteria may have acquired antibiotic resistance independently by contact with the antibiotics, or they may have acquired it by transformation. Natural antibiotic resistance of bifidobacteria (61) and lactobacilli (62) has been reported. To prevent the undesirable transfer of resistance or conferment of resistance to endogenous bacteria, probiotics should not carry resistance other than that required. Although special-purpose probiotics for use in combination with antibiotics have been developed through the introduction of multiple resistance to the bacteria (63), probiotics generally should not be designed to carry more resistance than is required for a specific purpose.

CONCLUSIONS

Assessment of the safety of probiotics from various angles is not a simple task. However, factors that can be determined in vitro are relatively easy to assess. The test item that has been attracting attention is whether the bacteria possess infectivity. Assessment of the ability to cause opportunistic infection is difficult. The acute and chronic toxicity tests probably provide circumstantial evidence. However, observations of the passage of bacteria across the intestinal barrier and invasion of the host body by translocation provide more direct data for determining infectivity. In the studies of Yamazaki et al, although translocation occurred in *B. longum*-monoassociated gnotobiotic mice, no harmful effects were observed; in contrast, the host immune system was activated. Platelet aggregation by bacteria is due to interactions among the high-molecular-weight substances on the bacterial surface, including proteins and carbohydrates. The relation of this phenomenon with infections including endocarditis remains to be studied. If translocation or infection starts from the moment of adhesion of the bacteria to the intestinal tract mucosa, then adhesiveness to intestinal epithelium, a required feature of probiotics, has to be discussed. The effects on the host of activities of glycosides and proteases that may degrade mucus need further study. Molecular biological studies of the bacteria isolated from infection sites and bacteria used in probiotics are required (64). The relation between the genetic characteristics of the bacteria and the type of infection, or the possibility of strain-specific infection, requires further studies.

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