Stimulation of the Immune System by Lactic Acid Bacteria

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Probiotic bacteria are important functional foods capable of improving consumer health. Although most probiotic bacteria are derived from human intestinal flora, they are not indigenous to all individuals. It is very likely that probiotic bacteria are seen as exogenous or foreign antigens by the gut immune system. In research on orally administered lactic acid bacteria, it was found that serum antibody responses were induced not only against the whole bacterial cell, but also against cytoplasmic components. Mucosal immune responses were induced against cytoplasmic antigens and may be of relevance in the development of mucosal vaccines using lactic acid bacteria. These bacteria also exert an adjuvant effect on the mucosal IgA response against not only pathogenic organisms, but also dietary antigens. Furthermore, they enhance systemic immune responses such as phagocytic activity and serum antibody production. These biological responses have been shown in human studies and have formed the basis for oral bacteriotherapy with probiotic bacteria. Since the immunopotentiating activity of lactic acid bacteria is strain-dependent, it is important that special efforts be directed to selection criteria used for the identification of biologically active strains with probiotic function.

Key words: lactic acid bacteria; probiotics; gut-associated lymphoid tissues; mucosal IgA response; immunopotentiating activity

INTRODUCTION

When we ingest food, most dietary components such as protein, fat, and carbohydrate are digested and absorbed in the gut. Thus the gut was believed to be only one part of the alimentary tract. However, the recent enormous progress in the study of the mucosal immune system has highlighted the gut as the largest immune organ in the body (31). To prevent a host from invasion by microbe and dietary antigen, the gut has lymphoid tissues such as Peyer's patches, mesenteric lymph nodes, lamina propria, and intraepithelial lymphocytes, which are referred to as gut-associated lymphoid tissues (GALT). GALT play a key role in the production of intestinal IgA responses to pathogenic microorganisms and in the induction of systemic unresponsiveness (oral tolerance) to substantially innocuous food proteins, thereby contributing to adaptation for the host to the environmental antigens (4).

A growing interest in health and diet has recently produced the concept of functional foods, and many food products designed to provide health benefits have been developed (46). Probiotics are among them and are defined as live microbial food supplements that beneficially influence the host by improving its intestinal microbial balance. Their purported benefits are the

maintenance of normal intestinal and urogenital microflora, alleviation of lactose intolerance, reduction of serum cholesterol levels, anticarcinogenic activity, stimulation of the immune system, and improved nutritional value of food (47). Although fewer human studies have been done compared with other benefits, the efficacy of probiotics on stimulation of the immune system is thought to be very promising. This is because most probiotics contain lactic acid bacteria derived from the human gastrointestinal microflora that are closely associated with the development and stimulation of GALT. In fact, there is increasing experimental evidence to support the effect of orally administered lactic acid bacteria on the immune system. Furthermore, stimulation of the host immune system is suggested to be one possible mechanism providing protection against diarrhea and tumor by lactic acid bacteria. Table 1 shows selected examples of lactic acid bacteria, including studies on parenteral administration (37). The present review summarizes data concerning the stimulation of the immune system by lactic acid bacteria.

IMMUNE RESPONSE TO ORALLY ADMINISTERED LACTIC ACID BACTERIA

Although most probiotic strains are derived from human intestinal flora, they are not indigenous to all individuals, and their ability to colonize the intestine differs with each one. In fact, it was reported that exogenous bifidobacteria and lactobacilli could colonize the host transiently (1, 25). Furthermore, lamina propria

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Table 1. The effect of viable and nonviable probiotic bacteria on the immune system (37).

Effect	Administration	Microorganism	Viable	Nonviable	Reference
<i>In vivo</i> humans					
Incidence of nasal allergies	Oral	Yogurt	Young subjects < 1 day ($p < 0.05$ to c) Elderly subjects < 7 days ($p < 0.05$ to c) 10/12 patients ($p = 0.002$ to nv)	Young subjects 2 days ($p < 0.05$ to c) Elderly subjects > 12 days ($p > 0.05$ to c) 2/13 patients	Trapp et al. (56)
Specific antibodies against rotavirus	Oral	<i>Lactobacillus</i> GG			Kaila et al. (18)
<i>In vivo</i> animals					
Adjuvant activity	Parenteral	<i>L. plantarum</i>	10^7 specific antigen producing cells ($p > 0.05$ to c)	2×10^7 specific antigen producing cells ($p < 0.03$ to v)	Bloksma et al. (2)
Adjuvant activity	Oral	Yogurt	$1.5\text{--}2 \times$ higher titres ($p < 0.02$ to c)	$1.5\text{--}2.5 \times$ higher titres ($p < 0.001$ to c)	Portier et al. (43)
Adjuvant activity	Oral	<i>L. casei</i> <i>L. acidophilus</i> <i>L. bulgaricus</i>	$3.5\text{--}4.5 \times$ stronger delayed type hypersensitivity response	<i>L. casei</i> $4.5 \times$ stronger response rest 'similar to control'	Perdigon et al. (39)
Increase in IgG2a levels upon vaccination	Oral	Yogurt	529 mg/dl ($p < 0.05$ to c)	174 mg/dl ($p > 0.05$ to c)	Conge et al. (6)
Adjuvant activity against <i>S. typhimurium</i>	Oral	<i>L. casei</i>	$1.3 \times$ higher intestinal anti- <i>Salmonella</i> IgA ($p > 0.05$ to c)	'Similar to control'	Perdigon et al. (38)
Protection from <i>S. typhimurium</i> infection	Oral	Yogurt	Mortality 53% ($p < 0.01$ to c)	Mortality 71% ($p > 0.05$ to c)	de Simone et al. (7)
Stimulation of reticulo-endothelial system	Parenteral	<i>L. plantarum</i>	Phagocytic index 0.82 ($p < 0.01$ to c) NK cell induction +62% lymph node response $7.4 \times$ ($p < 0.05$ to nv) <i>Listeria</i> clearance from spleen $2.5 \times 10^4\text{--}1.5 \times 10^5$ CFU ($p < 0.05$ to c)	Phagocytic index 0.78 ($p < 0.01$ to c) NK cell induction +55–85% lymph node response $5.6 \times$ <i>Listeria</i> clearance from spleen $10^4\text{--}1.2 \times 10^5$ CFU ($p < 0.05$ to c)	Bloksma et al. (3)
Tumour size, survival rate	Parenteral	<i>B. infantis</i>	75%	67%	Kohwi et al. (24)
Tumour size, survival rate	Parenteral	<i>B. adolescentis</i>	67%	43%	Kohwi et al. (24)
30-Day survival after whole-body γ -irradiation	Parenteral	<i>L. casei</i> YIT 9018	Not tested	80–100% ($p < 0.01$ to c)	Tsuneoka et al. (57)
Reduction of transplantable tumour growth	Oral	<i>L. casei</i> YIT 9018	Not tested	–27% ($p < 0.001$ to c)	Matsuzaki and Yokokura (30)
Stimulation of splenocytes	Oral	<i>L. casei</i> YIT 9018	Not tested	$2.8\text{--}10.9 \times$ higher cytolytic activity ($p < 0.001$ to c)	Matsuzaki and Yokokura (30)
Reduction of secondary tumour growth	Oral	<i>L. casei</i> YIT 9018	$25\text{--}34\%$ smaller tumour ($p > 0.05$ to c)	No effect	Kato et al. (21)
Increase in serum IgG2a, IgM, and oxidative metabolism and proliferation of lymphocytes	Oral	Yogurt	IgG2a 85 mg/dl ($p < 0.05$ to c) IgM 31 mg/dl ($p < 0.05$ to c) Proliferative response $1.6 \times$ ($p < 0.01$ to c) Oxidative metabolism $1.4 \times$ ($p < 0.01$ to c)	IgG2a 89 mg/dl ($p < 0.05$ to c) IgM 31 mg/dl ($p < 0.05$ to c) Proliferative response as control oxidative metabolism $1.5 \times$ ($p < 0.01$ to c)	Vesely et al. (58)

Table 1. (continued)

Effect	Administration	Microorganism	Viable	Nonviable	Reference
<i>In vivo</i> animals					
Production of IFN α and β	Parenteral	<i>L. bulgaricus</i>	64 IU/ml	Heated < 4 IU/ml irradiated 350 IU/ml	Solis-Pereyra et al. (50, 51)
Production of interferon- γ by T-cells	Oral	Yogurt	20 U/ml ($p > 0.05$ to c)	1.7 U/ml	Halpern et al. (14)
Proliferative response of Peyer's patch cells	Oral	Yogurt	50 cpm/min ($p < 0.001$ to c)	40 cpm/min ($p < 0.01$ to c)	de Simone et al. (8)
Immunoglobulin expressing cells in Peyer's patches	Oral	Yogurt	51% after 7 and 14 days ($p < 0.01$ to c)	48% after 14 days ($p < 0.05$ to c)	de Simone et al. (9)
<i>In vitro</i>					
Induction of IFN α in murine macrophage cultures		<i>L. gasseri</i>	Not tested	42.6 IU/ml (control 9.7 IU/ml)	Kitazawa et al. (23)
Induction of TNF- α and IL-6 in human peripheral blood mononuclear blood leukocytes		<i>L. rhamnosus</i> E509 <i>L. rhamnosus</i> GG	TNF- α 75–95 ng/ml ($p < 0.001$ to nv) IL-6 6 ng/ml ($p < 0.01$ to nv) + 200% ($p < 0.001$ to c)	TNF- α Glutaraldehyde fixed 15 ng/ml IL-6 Glutaraldehyde fixed 2 ng/ml	Miettinen et al. (32)
INT-reductase activity of human neutrophils and guinea-pig macrophage		<i>E. faecium</i>		'No differences in effect of viable and heat-inactivated'	Ebringer et al. (12)

c, control (no bacteria); nv, nonviable; v, viable.

mononuclear cells were shown to be tolerant to bacteria residing in autologous but not heterologous human intestine (11). Therefore it is important to examine the immune response to orally administered lactic acid bacteria, even though probiotic bacteria are generally recognized as safe for human and animal consumption. Since lactic acid bacteria are candidates for antigen delivery vehicles for oral immunization (44), this information will also be useful for the development of the mucosal vaccine.

With regard to the immune response to lactic acid bacteria, serological studies for bacterial classification have been performed on many strains of lactobacilli, and these cellular fractions as teichoic acid and polysaccharide were found to be reactive to the antiserum from rabbits immunized parenterally with bacterial cells (20). However, only a few studies have been made on the immune response to lactic acid bacteria administered orally. Yasui et al. (60) reported immunogenicity of *Bifidobacterium breve* and change in antibody production in Peyer's patches after oral administration. Immunogenicity of *B. breve* was weak, and temporary serum antibody response to the organism was detected in mice fed these bacteria for more than 33 days. Antibody production against *B. breve* by Peyer's patch cells was also detected transiently in them. Furthermore, such weak immunogenicity of orally ingested lactic acid bacteria was shown in human study (5).

Because lactic acid bacteria have highly complex structures with many immunogenic epitopes, it is further important to determine which components of the whole bacteria cell are responsible for the serum antibody response to clarify the interaction of lactic acid bacteria with the immune system. The authors investigated the antibody response of mice to cell wall and cytoplasmic fractions of orally administered lactic acid bacteria (54). In mice fed with *B. longum* for more than 8 weeks, an antibody response to the cytoplasm of *B. longum* was detected, but not to the cell wall. On the other hand, in mice fed with *Lactobacillus acidophilus* for more than 6 weeks, an antibody response to the cytoplasm and to the cell wall of *L. acidophilus* was detected. Moreover, feeding each organism for 2 weeks enhanced the proliferative response of Peyer's patch cells to the cell fraction against which the serum antibody was detected. This suggests that the immune response to lactic acid bacteria differs with the bacterial strains tested and that cytoplasmic fractions of lactic acid bacteria are associated with the interaction between dietary lactic acid bacteria and the host immune system. It is interesting that a Western blot analysis of proteins derived from the cytoplasmic fractions of *B. longum* and reacted with sera from mice orally immunized with *B. longum* defined several proteins that are not recognized by mice parenterally immunized with *B. longum* (Fig. 1). Similarly, Norton et al. (36) de-

Fig. 1. Western blot showing the reactivity of sera from mice fed for 12 weeks with or immunized parenterally with *B. longum* to the cytoplasmic fractions of *B. longum* cell. Lane 1: sera from mice fed for 12 weeks with *B. longum*; Lane 2: sera from mice immunized parenterally with *B. longum*. Size markers are indicated.

tected serum antibody responses to subcellular protein fractions derived from *L. lactis* following oral inoculation of *L. lactis* by a Western blot analysis. The examination of these proteins may give clues to the development of mucosal vaccine.

EFFECT OF ORALLY ADMINISTERED LACTIC ACID BACTERIA ON THE IMMUNE RESPONSE TO ENVIRONMENTAL ANTIGENS

The production and expression of secretory IgA, the most abundant immunoglobulin in mucosal secretions, is central to the gut immune function (31). Thus to protect the host against an invasion of pathogenic organisms, antigens, and toxic substances from the gut lumen, the enhancement of specific and nonspecific IgA response appears to be necessary for the efficacy of probiotics. In this regard, several investigations suggest that orally administered lactic acid bacteria increase the mucosal IgA response to foreign substances.

For example, Perdigon et al. (40) found that intestinal IgA antienteropathogen levels were higher in mice previously fed for 2 days with *L. casei* than in control mice after *Salmonella typhimurium* or enterotoxigenic *Escherichia coli* challenge. Yasui et al. (59) showed that the level of antirotavirus IgA in the milk of mouse dams fed *B. breve* was significantly higher than in the

milk of control dams after rotavirus challenge. Furthermore, Tejada-Simon et al. (55) indicated that the fecal IgA anticholera toxin level was much higher in mice fed yogurt with bifidobacteria and *L. acidophilus* than in mice fed skim milk. Since these pathogenic organisms and toxin are associated with a high rate of infant mortality and morbidity, especially in developing countries, dietary lactic acid bacteria are expected to contribute to the host defense also in humans. In fact, infants fed fermented milk containing *Lactobacillus* GG showed an increase in the specific IgA response to rotavirus and earlier recovery from rotavirus diarrhea, compared with the control group (19).

On the other hand, more and more infants are suffering from food allergies in developed countries. It is currently recognized that some dietary protein antigens may remain partly intact in the gastrointestinal tract and cause most of the allergic reactions (4). This suggests that mucosal defense against dietary protein antigens may be important for the prevention of allergic reactions to them. However, few reports have been made on the effects of lactic acid bacteria on the host defense against luminal dietary antigens. Thus the authors investigated the effects of lactic acid bacteria on the mucosal IgA response of mice to β -lactoglobulin (β -LG), a whey protein antigen often ingested with lactic acid bacteria in dairy foods such as yogurt (53). The anti- β -LG IgA level in tissue extracts of the small intestinal wall was much higher in mice fed the *B. longum* diet for 2 weeks than in control mice. Furthermore, the *in vitro* IgA response of Peyer's patch (PP) cells to β -LG was detected only when PP cells from mice fed *B. longum* were assayed, not when those from control mice were assayed. These results suggest that orally ingested lactic acid bacteria have an adjuvant effect on the mucosal IgA response to dietary protein antigens as well as to pathogenic organisms. Our findings compare favorably with the observations of Fukushima et al. (13), who have shown that the level of anti- β -LG IgA in fecal extracts was significantly higher in mice fed bifidobacteria than the level in control mice.

Recently, Sudo et al. (52) reported the role of intestinal bacterial flora in oral tolerance to the IgE response. The reconstitution of intestinal flora of germfree mice with *B. infantis* restored the susceptibility of the IgE response to oral tolerance induction only when the bacteria were inoculated into these mice at the neonatal stage, but not at an older age. Thus they suggested that exposure to intestinal microflora at the neonatal stage is required if GALT is to become fully developed and generate competent regulatory cells. In accordance with

this, Sepp et al. (49) have shown that the prevalence of atopic disease is low in Estonia and high in Sweden and that the differences in intestinal microflora include more lactobacilli in the former.

Taken together, probiotic bacteria have been suggested to promote endogenous barrier mechanisms in a host. This notion is supported by the observations of Majamaa and Isolauri (27), who applied probiotic bacteria to the treatment of food allergy and alleviated the intestinal inflammation in patients.

Furthermore, it is well known that orally administered lactic acid bacteria increase the systemic immune response in a host (41). Especially, there are accumulating data to support their enhancing effect on phagocytic activity and antibody production also in man (10, 17, 48). These studies seem to form the basis for oral bacteriotherapy with probiotic bacteria.

STRAIN DEPENDENCY OF THE IMMUNOPOTENTIATING ACTIVITY OF LACTIC ACID BACTERIA

Since most lactic acid bacteria such as lactobacilli and bifidobacteria are normal intestinal microflora of humans and generally recognized as safe, little attention was paid previously to the difference of probiotic effect among species or strains. However, several different *in vitro* assays have been developed to screen new strains of bacteria for their ability to act as probiotics and to provide a health benefit to the host (45). This is so also for the selection of strains with high immunopotentiating activity.

Yasui et al. (61) screened 120 strains of bifidobacteria isolated from human feces by using the PP cell culture method and found three strains of bifidobacteria inducing large quantities of IgA among them. Lee et al. (26) investigated the mitogenic activity of bifidobacteria and lactobacilli using spleen, thymus, and PP cells and indicated that the occurrence of immunopotentiating activity was dependent on the properties of individual strains instead of on the species or microbial culture conditions. Furthermore, Marin et al. (28, 29) evaluated the effect of streptococci, lactobacilli, and bifidobacteria on cytokine production by using a clonal macrophage and T-cell lines and selected several strains that stimulated cytokine production strongly. Therefore a knowledge of the identity of probiotic strains used seems to be a prerequisite for the development of probiotic bacteria that most effectively enhance the immune response.

In the dairy industry, *Lactobacillus delbrueckii* subsp. *bulgaricus* is historically one of the most widely used and best characterized bacteria strains. However, little

information is available on the strain dependence of the immunopotentiating activity for *L. delbrueckii* subsp. *bulgaricus*. Thus the authors screened 90 strains of this bacterial species for the proliferative response of spleen and β -LG-primed lymph node cells (34). The immunopotentiating activity varied greatly between strains. Among them, several, including 1023, showed strong activity. Furthermore, strain 1023 had the strongest mitogenic activity for PP cells and induced IgA antibody production by PP cells as strongly as the *B. longum* strain that previously had been shown to have an oral adjuvant effect. These results suggest that *L. delbrueckii* subsp. *bulgaricus* strains such as 1023 may be useful for the production of fermented milk with a more beneficial effect on the host immune system.

What components are responsible for the strain dependence of the immunopotentiating activity of lactic acid bacteria? Although it is generally recognized that the immunomodulatory factor derived from Gram-positive bacteria is peptidoglycan contained in the cell wall (15), there have been controversial results on the immunoactive fractions of lactic acid bacteria. Indeed, Namioka et al. (35) demonstrated that the number of IgA-bearing cells in the lamina propria of the small intestine was significantly larger in the piglets fed with peptidoglycan from bifidobacteria than the control piglets. However, Morata de Ambrosini et al. (33) observed that the oral administration of peptidoglycan from *L. casei* did not produce an IgA increase in mouse intestinal fluid, whereas the whole bacterial cell did. Furthermore, Hatcher and Lambrecht (16) showed that cell-free extracts from *L. acidophilus* and *B. longum* enhanced macrophage activity, which is consistent with our findings that the oral administration of these organisms induced a proliferative response of PP cells to their cytoplasm (54). Also with *L. delbrueckii* subsp. *bulgaricus* 1023, a mitogenic response of spleen and PP cells was detected to both the cell wall and the cytoplasm of the strain (Table 2). Therefore additional components not included in the cell wall fraction are hypothesized to be involved in immunostimulation by lactic acid bacteria.

Besides bacterial products such as peptidoglycan and polysaccharide, recent studies indicate that bacterial DNA stimulates B cells, T cells, and macrophages to proliferate, secrete antibodies, and/or produce cytokines (42). This immunopotentiating DNA is reported to contain six base pair motifs consisting of an unmethylated CpG dinucleotide flanked by two 5'-purines and two 3'-pyrimidines. In contrast with this, a novel DNA motif inducing mitogenic activity has been found in DNAs

Table 2. Mitogenic response of spleen and PP cells to the cell fractions of *L. delbrueckii* subsp. *bulgaricus* 1023^a.

Sample	Spleen	PP
None (control)	1.1 ± 0.1	1.1 ± 0.3
Cell wall	6.0 ± 0.5	13.4 ± 2.0
Cytoplasm	7.2 ± 0.5	7.7 ± 0.6

^aSpleen and PP cells were cultured with the cell fractions of *L. delbrueckii* subsp. *bulgaricus* 1023 (100 µg/ml) for three days, and [³H] thymidine uptake was then assessed by 20 hr pulsing and is expressed as the mean of the stimulation index. Error bars indicate standard deviations for three wells.

from lactic acid bacteria (22). Thus it seems tempting to speculate that such oligodeoxynucleotides might be a candidate substance for immunoactive components in cell-free extracts of lactic acid bacteria. Further study will be needed to determine which cell components of lactic acid bacteria are responsible for the strain dependence of the immunopotentiating activity.

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