

## BIFIDOBACTERIA AND LACTOBACILLI EXHIBITED DIFFERENT MITOGENIC ACTIVITY ON MURINE SPLENOCYTES

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**ABSTRACT:** *Forty-one strains of Bifidobacterium and Lactobacillus were tested for their ability to induce the proliferation of murine splenocytes using the MTT assay. The tested bifidobacteria induced the apparent proliferation of the murine splenocytes in a strain-dependent manner, while none of the tested lactobacilli showed any mitogenic activity. The bifidobacteria from allergic infants stimulated the proliferation of murine splenocytes more than those from healthy subjects. On the other hand, no significant differences were observed in the ability among the selected bifidobacteria and lactobacilli to alter Concanavalin A stimulated proliferation of murine splenocytes. These results suggest that lactobacilli and bifidobacteria may, at least partly, possess a different potential in the way by which they can affect human immune responses.*

**KEY WORDS:** Bifidobacteria, Lactobacilli, Probiotics, Proliferation

**ABBREVIATIONS USED:** CFU, colony-forming units; Con A, Concanavalin A; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; PBS, phosphate buffered saline; SDS, Sodium dodecyl sulfate

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### INTRODUCTION

More than 400 species of bacteria are estimated to reside in the human gastrointestinal tract these endogenous bacteria comprise the complex human intestinal microflora (Simon and Gorbach, 1996; Mitsuoka 1980). The intestinal microflora contributes to the development and function of the mucosal barrier and

colonization resistance in the human intestine, which protects the host from various infections and aberrant antigen overload. The underlying mechanisms proposed for these protective effects may include production of antimicrobial substances, competition for nutrients and adhesion receptors (Roberfroid et al., 1995). Recently, the increase in the knowledge on the cross talk between the endogenous bacteria and the gut-associated lymphoid tissue (GALT) suggests that the intestinal microflora may be one of the fundamental boosters for both local and systemic immune responses (Isolauri, 2001; Cross and Gill, 2001). Therefore, the maintenance of a balanced human intestinal microflora is considered beneficial for health and well being.

Bifidobacteria and lactobacilli are among the predominant groups of the normal microflora of the human intestine (Mitsuoka, 1980; Hammes and Vogel, 1995). The presence of these bacteria in the human intestine has been considered to be one of the most important aspects of a healthy intestinal microflora (Hammes and Vogel, 1995; Ballongue, 1998; Saavedra et al., 1994). Therefore, many attempts have been made to increase their number in the intestine by administration of certain probiotic strains or prebiotics including oligo- and polysaccharides that stimulate the growth and activity of bifidobacteria and lactobacilli (Mitsuoka, 1984). Bifidobacteria and lactobacilli are the most prospective candidate for probiotics use. Many studies indicate that bifidobacteria and lactobacilli may possess many similarities in their health promoting effects, for instance, shortening and preventing diarrhea caused by various types of pathogenic agents, and stimulation of human immune responses (Hammes and Vogel, 1995; Mitsuoka, 1984). However, bifidobacteria and lactobacilli belong to different taxonomic groups with significant differences in many phenotypic and genotypic properties (Mitsuoka, 1984). Although both are natural residents of the human intestine, a diverse ecological distribution and quantity in the intestinal microflora exist between these bacteria (Mitsuoka, 1984). Therefore, it is of interest to characterize the interactions of these bacteria with the host immune system, which will promote their use more effectively for specific health-promoting targets.

In the present study, 41 strains of bifidobacteria and lactobacilli, isolated from dairy food and human intestine, were tested for their potential to induce the proliferation of murine splenocytes in the MTT assay. Furthermore, six strains of the tested bacteria

were chosen to investigate their influences on Con A stimulated proliferation of mouse whole spleen cells.

## MATERIALS AND METHODS

### Mouse spleen cells

Male C57BL/6 (6 week old) mice were purchased from Japan Charles River, Inc. (Yokohama, Japan). The mice were kept in plastic cages, and were given a standard diet (MF-1, Oriental Yeast Co. Ltd., Tokyo, Japan), and were allowed free access to water for 1 to 2 weeks before the start of the experiments. The temperature and humidity were controlled at  $24^{\circ}\text{C} \pm 1$  and  $55 \pm 10\%$ , respectively.

To obtain spleen cell preparation, mice were sacrificed and their spleen cells were aseptically removed. Single cell suspensions were prepared by gently teasing each spleen in a 5 cm petridish with 5 ml of RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 10 % NuSerum (Becton Dickinson, Two Dak Park, Bedford, MA, USA), and 100  $\text{ig ml}^{-1}$  streptomycin, 100  $\text{ig ml}^{-1}$  penicillin, 10 mM HEPES and 50  $\text{imM}$  2-mercaptoethanol. The cells were washed three times with the same RPMI 1640 medium, and resuspended in RPMI 1640 medium at a concentration of  $5.0 \times 10^6$  cells  $\text{ml}^{-1}$ .

### Bacterial preparation

As test bacteria, 30 strains of bifidobacteria and 11 *Lactobacillus* strains, stored at the Microbiological Laboratory of Takanashi Milk Products Co., Ltd (Yokohama, Japan) were used. A probiotic strain, *L. rhamnosus* GG (ATCC 53103) was obtained from Valio Ltd. (Helsinki, Finland). Bifidobacteria and lactobacilli were pre-cultured two or three times in Gifu anaerobic medium (GAM; Nissui Pharmaceutical Co., Ltd.) and de Man, Rogosa and Sharp broth (MRS broth; Difco Laboratories, Detroit, Michigan, USA) under anaerobic or aerobic condition respectively, for 18 to 24 hr. at  $37^{\circ}\text{C}$ . After incubation, the bacteria were collected by centrifugation and washed three times with phosphate buffered saline (PBS; pH 7.2, 10 mM phosphate). Subsequently, the bacteria were resuspended in RPMI 1640 medium at a concentration of  $10^8$  CFU  $\text{ml}^{-1}$ , and heat-inactivated at  $65^{\circ}\text{C}$  for 30 min after the sonic treatment with ultrasonic processor (Sonicator No.5202, Ohtake Tokyo, Japan). These bacterial preparations were kept at  $-70^{\circ}\text{C}$  until use.

### Proliferation assay

Mitogenic activities were determined by assessing MTT reduction following the method described by Morusaki et al., (1999) with some modifications. Briefly, spleen cells were divided onto a 96 well flat-bottomed plate (Nalge, Nunc International, Rochester, N.Y., USA) with  $5 \times 10^5$  cells  $\text{ml}^{-1}$  in each well. To wells, 100  $\mu\text{l}$  of heat-inactivated bacteria were added. Con A (Sigma, St. Louis, USA) was added at a concentration 2  $\text{ig well}^{-1}$  to promote the proliferation of T cells. The culture was incubated at  $37^{\circ}\text{C}$  in an atmosphere of air -5 %  $\text{CO}_2$  for 48 hr. At 3 hr. prior to terminating the culture, a 0.5 % MTT solution, dissolved in RPMI 1640 medium, was added to each well (10  $\mu\text{l well}^{-1}$ ). The cell cultivation was terminated by adding 50  $\mu\text{l well}^{-1}$  of SDS solution (20 % SDS in 0.03 N HCl) into each well. OD at 550 nm and OD at 630 nm were measured by using a microplate

reader (ImmunoMin NJ-2300, InterMed, Tokyo, Japan). The percentage of proliferation was calculated as follows:

$$\text{Proliferation (\%)} = (\text{Abs } 550\text{-}630 \text{ with tested bacteria} - \text{Abs } 550\text{-}630 \text{ of control}) \times 100\%$$

### Statistical analysis

The Kruskal-Wallis test and the two-tailed independent Student's *t*-test were used for a comparison of differences in proliferation of mouse whole spleen cells between bifidobacteria isolated from allergic infants and those from healthy subjects. Fisher's exact test was used to determine the difference in proliferation between lactobacilli and bifidobacteria.

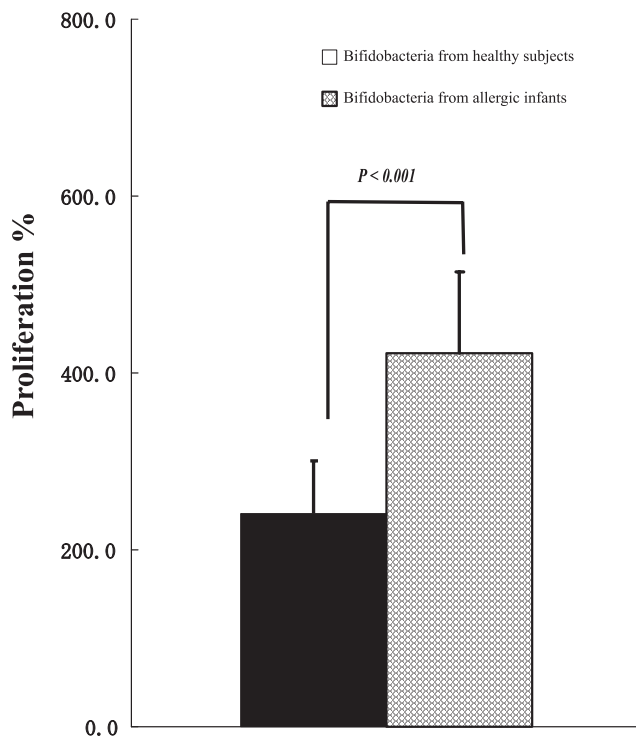
## RESULTS

All of the tested bifidobacteria induced proliferation of mouse whole spleen cells in a strain-dependent manner (Table 1). However, non of the tested lactobacilli caused any proliferation of the spleen cells compared to bifidobacteria (Table 1). The tested bifidobacteria isolated from allergic infants induced proliferation of murine splenocytes more than did those isolated from healthy subjects ( $p < 0.001$ ) (Fig. 1). Four tested bifidobacteria and two tested lactobacilli with different mitogenic effect were chosen for the examination of their effects on Con A stimulated proliferation of the murine splenocytes. All of the tested bacteria suppressed the Con A stimulated proliferation of the mouse spleen cells (Fig. 2). The strongest suppressive effect on Con A stimulated proliferation of the mouse spleen cells was observed with *L. rhamnosus* GG..

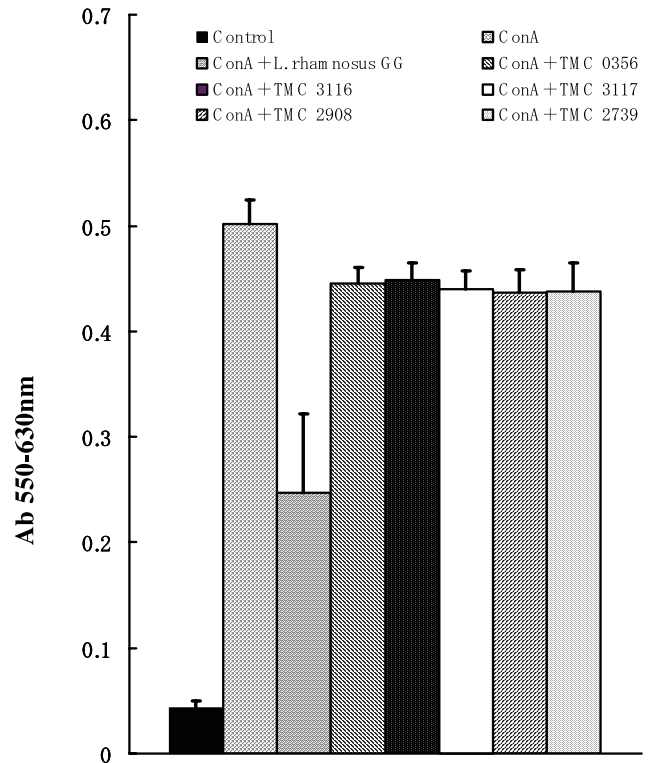
**Table 1. Proliferation of murine whole spleen cells after exposure to lactobacilli and bifidobacteris**

Species Name	Strain No	Origine	Proliferation*
<i>Lactobacillus acidophilus</i>	TMC 0313	Dairy food	-
<i>L. acidophilus</i>	TMC 0356	Human intestine	±
<i>L. casei</i>	TMC 0402	Dairy food	-
<i>L. casei</i>	TMC 0409	Dairy food	-
<i>L. rhamnosus</i>	TMC 0503	Dairy food	-
<i>L. rhamnosus</i>	TMC 0510	Dairy food	-
<i>L. rhamnosus</i>	GG	Human intestine	-
<i>L. rhamnosus</i>	TMC 0517	Dairy food	±
<i>L. casei</i>	TMC 1001	Human intestine	-
<i>L. casei</i>	TMC 1002	Human intestine	-
<i>L. casei</i>	TMC 1003	Human intestine	-
<i>Bifidobacterium longum</i>	TMC 2607	Dairy food	++
<i>B. longum</i>	TMC 2608	Human intestine	++
<i>B. longum</i>	TMC 2609	Human intestine	++
<i>B. longum</i>	TMC 2614	Allergic infant	+++
<i>B. longum</i>	TMC 2615	Allergic infant	+++
<i>B. adolescentis</i>	TMC 2701	Human intestine	++
<i>B. adolescentis</i>	TMC 2704	Human intestine	++
<i>B. adolescentis</i>	TMC 2705	Human intestine	++
<i>B. adolescentis</i>	TMC 2718	Allergic infant	++
<i>B. adolescentis</i>	TMC 2720	Allergic infant	+++
<i>B. adolescentis</i>	TMC 2721	Allergic infant	+++
<i>B. adolescentis</i>	TMC 2723	Allergic infant	+++
<i>B. adolescentis</i>	TMC 2736	Allergic infant	+++
<i>B. adolescentis</i>	TMC 2737	Allergic infant	+++
<i>B. adolescentis</i>	TMC 2938	Allergic infant	+++
<i>B. adolescentis</i>	TMC 2739	Allergic infant	+++
<i>B. infantis</i>	TMC 2906	Human intestine	+++
<i>B. infantis</i>	TMC 2908	Human intestine	++
<i>B. pseudocatenulatum</i>	TMC 3001	Human intestine	++
<i>B. bifidum</i>	TMC 3101	Dairy food	+++
<i>B. bifidum</i>	TMC 3108	Human intestine	++
<i>B. bifidum</i>	TMC 3115	Human intestine	++
<i>B. bifidum</i>	TMC 3116	Human intestine	+++
<i>B. bifidum</i>	TMC 3117	Human intestine	+++
<i>B. breve</i>	TMC 3207	Human intestine	+++
<i>B. breve</i>	TMC 3217	Dairy food	+++
<i>B. breve</i>	TMC 3218	Dairy food	++
<i>B. breve</i>	TMC 3219	Dairy food	++
<i>B. animalis</i>	TMC 5101	Dairy food	+++
<i>Bifidobacterium sp.</i>	TMC 3524	Allergic infant	+++

\* Results were expressed as: The control was made to be 100% (-) <100. (±): 100 - 150, (+): 150-300, (++) : 300-500, (+++): 500<



**Fig. 1.** Proliferation of murine splenocytes stimulated by Bifidobacterium



**Fig. 2.** Proliferation of murine splenocytes stimulated by Concanavaline A in the presence with bifidobacteria and lactobacilli

## DISCUSSION

We previously investigated the ability of bifidobacteria and lactobacilli to induce the secretion of cytokine production by human enterocyte like cells. It was observed that some bifidobacteria caused a slight induction of the pro-inflammatory cytokines, Interleukin (IL)-6 and IL-8, although these cytokines were not detected after exposure to lactobacilli (Morita et al., 2002). Similar differences between bifidobacteria and lactobacilli were found in their ability to activate murine macrophages cells, in which bifidobacteria rather than lactobacilli induced the more cytokines production (Morita et al., 2002a; He et al., 2002b). In the present study, we compared the mitogenic activities of bifidobacteria and lactobacilli on the proliferation of murine splenocytes, to extent the understanding on the differences in the immunomodulatory effects of these two genera. Interestingly, bifidobacteria but not lactobacilli exhibited pronounced mitogenic activities on murine splenocytes, inducing a significant proliferation of whole spleen cells. Mitogenic activities are generally considered to be one of the important properties of immune modulators, and have been used to evaluate the immunomodulating activities of lactic acid bacteria (Kado-oka et al., 1991; Kitazawa et al., 1992; Kakeda et al., 1997; Ko et al., 1999). The results of the present study indicate that differential immune modulatory effects may exist between bifidobacteria and lactobacilli, suggesting that bifidobacteria may be stronger

boosters of host immunity. These results also indicate that the different ability between bifidobacteria and lactobacilli to stimulate cytokine production by enterocytes and macrophage cells may be linked to their different mitogenic activities.

Although bifidobacteria are often grouped together with lactobacilli as lactic acid bacteria, significant differences exist between these two genera both taxonomically and ecologically (Hammes and Vogel, 1995; Mitsuoka, 1984). Lactobacilli are main residents of the small bowel, and bifidobacteria are normal inhabitants of the human colon. Compared to the upper digestive tract, the microflora in the human colon are more diverse and complex, containing more facultative anaerobic gram-negative bacteria (Hammes and Vogel, 1995; Mitsuoka, 1984). These bacteria, mainly opportunistic or endogenous infective agents, have been found to be the strong triggers of anti-inflammatory cytokine production by macrophages and monocytes (Hammes and Vogel, 1995). The strong mitogenic activities may be one of acquired properties for bifidobacteria for their ecological distribution. Therefore, the function of various endogenous lactic acid bacteria can not replace each other, rather than a good balance between endogenous bacteria is the important for a healthy microflora. Furthermore, 11 strains of *B. adolescentis* from allergic infants exhibited much stronger mitogenic effects on murine splenocytes than those from dairy foods, healthy infants or adults. These results are consistent with our previous findings that *B.*

*adolescentis* from the allergic infants may enhance more inflammatory responses than those from health infants and adults (He et al., 2002). *B. adolescentis* is a normal resident of the adults intestine (Mitsuoka, 1984). However, *B. adolescentis* was also isolated as predominant species in the fecal *Bifidobacterium* flora of allergic infants (He et al., 2001; Ouwehand et al., 2001). The results presented here emphasize again that abnormality in the species composition with age may negatively influence the homeostasis of human immunity. This suggests that the impact of the endogenous bifidobacteria on human immunity may be, at least partly, species dependent.

Lipoteichoic acid and peptidoglycan are major components of the cell wall of the gram-positive bacteria, which can be regarded as the gram positive analogues of lipopolysaccharide can activate polyclonal proliferation of lymphocytes including macrophage and B cells (Laman et al., 2002). Therefore, the observed proliferative responses to the tested bifidobacteria are considered to relate to the activities of B cells and macrophage in whole spleen cells. It is well known that the composition of the cell wall of bifidobacteria is significantly different from lactobacilli (Hammes and Vogel, 1995; Habu et al., 1987). The differences in the mitogenic activities of bifidobacteria and lactobacilli may, at least partly, be attributed to the differences in cell wall composition of these bacteria.

Commensal and pathogenic bacteria differ in the terms of their action on immune cells in the gut (Jiang et al., 1999; Rahimi et al., 1995). Bacteria and bacterial homogenates of the commensal gut microflora do not stimulate proliferation of mononuclear cells and play an important role in maintenance of hyporesponsiveness to foreign antigens (Sato et al., 1998). Pathogens, in contrast, activate mucosal immune cells and cause the proliferation of these cells, triggering an inflammatory reaction (Jiang et al., 1999; Rahimi et al., 1995). T cells are one of the critical immune competent cells involved in cell-mediated immune responses in the adaptive immune system (Abbas et al., 2001). Con A is a mitogen that can specifically stimulate the proliferation of T cells. In the present study, all of six selected tested bacteria including bifidobacteria and lactobacilli suppressed the Con-A stimulated proliferation of murine splenocytes. Different from the mitogenic activities, no significant differences were observed between bifidobacteria and lactobacilli in their suppressive effects to Con A stimulated proliferation of murine splenocytes. These results indicate that both bifidobacteria and lactobacilli may be beneficial in controlling T cell mediated hypersensitivity reaction by which they can contribute to human immune homeostasis.

*L. rhamnosus* GG is a well investigated probiotic strain, which possess many documented health-promoting effects in humans (Saxelin et al., 1998). The most recent prospective clinical studies indicated that this bacterium could effectively reduce the incidence of allergy and alleviate symptoms of allergic disease (Kalliomaki et al., 2001; Majamaa et al., 1997). Modulation of the immune system by this bacterium is thought to be behind of these beneficial health effects (Isolauri, 2001). In previous studies,

the hydrolysis of caseins with *L. rhamnosus* GG-derived enzymes was found to generate the molecules with the suppressive effect on lymphocytes proliferation (Sutas et al., 1996). Furthermore, such a suppressive effect by *L. rhamnosus* GG-degraded bovine casein on the proliferation of lymphocytes was observed to be limited to T cells (Pessi et al., 2001). In addition, the homogenates derived from *L. rhamnosus* GG were also found to possess anti-proliferative effects to phytohemagglutinin-induced proliferation of mononuclear cells (Pessi et al., 1999). The results in the present study confirmed and extended the previous findings, indicating that the cell components of *L. rhamnosus* GG can express anti-proliferative effect on T cells much stronger than other lactobacilli and bifidobacteria.

In conclusion, the current study has shown that the tested bifidobacteria stimulated the mitogenic activity of murine spleen cells, which lactobacilli do not. Further, bifidobacteria isolated from allergic infants stimulated the proliferation stronger than bifidobacteria from other origins. *L. rhamnosus* GG was shown to exhibit the strongest anti-proliferative effect. Different *Bifidobacterium* and *Lactobacillus* strains can therefore be expected to have very different effects on human the hosts immune system.

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