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Chapter 1

两歧双歧杆菌 BGN4

Bifidobacterium bifidum BGN4

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Journal of Food Protection, Vol. 63, No. 8, 2000, Pages 1133–1136

Binding of Aflatoxin B_1 to Bifidobacteria In Vitro

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MS 99-250: Received 18 August 1999/Accepted 22 February 2000

ABSTRACT

Aflatoxins are mycotoxins that cause health and economic problems when they contaminate food and feed. One potential method for reducing human health effects due to aflatoxin ingestion is to block uptake via binding by bacteria that either make up the normal gut flora or are present in fermented foods in our diet. These bacteria would bind aflatoxin and make it unavailable for absorption in the intestinal tract. Bifidobacteria comprise a large fraction of the normal gut flora, are thought to provide many probiotic effects and are increasingly used in fermented dairy products. These qualities targeted bifidobacteria for studies to determine if various strains of heat-killed bifidobacteria can bind aflatoxin B_1 (AFB_1) in vitro. The AFB_1 binding affinities of various strains of bifidobacteria, *Staphylococcus aureus*, and *Escherichia coli* were quantitated utilizing enzyme-linked immunosorbent and [^{3}H]AFB_1 binding assays. The bacteria analyzed were found to bind significant quantities of AFB_1 ranging from 25% to nearly 60% of the added toxin. The data also suggest that there are reproducible strain differences in AFB_1 binding capacity.

Aflatoxins are fungal secondary metabolites produced by a small number of aspergilli including *Aspergillus flavus* and *Aspergillus parasiticus (7)*. These fungi are ubiquitous and commonly contaminate foods and feeds including corn, peanuts, and tree nuts *(7, 23)*. There are as many as 18 different forms of aflatoxin and its metabolites *(16)*. Of these, aflatoxin B_1 (AFB_1) is the most prevalent and toxic *(7)*. In fact, AFB_1 is the most potent naturally occurring carcinogen known in certain animal models *(7, 17)*. It is currently under investigation as a suspected hepatocarcinogen in humans *(17)*. Contamination of animal feed may also pose human health problems due to the occurrence of aflatoxin M1, a less potent, hydroxylated form of AFB_1 excreted in cow's milk *(18, 23)*.

Many pre- and postharvest methods for aflatoxin control are currently being investigated. Postharvest methods include the removal or degradation of aflatoxin in foods and feeds by extraction, bentonite clay, UV light, bacteria, fungi, and algae *(3, 7, 23)*. One postharvest mechanism under study is the removal of aflatoxin in the gut by lactic acid bacteria *(9, 14, 24)*. Previous work has focused on lactobacilli; however, our attention has turned toward bifidobacteria. Bifidobacteria are excellent candidates for binding studies because they are one of the most abundant group of microorganisms found in the gut, with 3 to 4 logs more cells per gram of feces than lactobacilli *(1, 22)*. Bifidobacteria are gram-positive, rod-shaped, lactic acid-producing microorganisms. The reported health and nutritional benefits of bifidobacteria include composition control of intestinal flora via colonization and pH *(2, 10, 19)*, stimulation of immune functions *(12, 20)*, synthesis of B-complex vitamins *(6)*, improvement of lactose tolerance and digestibility of milk products, antitumorigenic activity, reduction of serum cholesterol levels, and absorption of calcium *(11, 19)*. The antimicrobial effects of bifidobacteria inspired its use in dairy products leading to more than 70 dairy products containing bifidobacteria produced worldwide including sour cream, buttermilk, yogurt, powdered milk, cookies, and frozen desserts *(5, 13, 19)*. The aim of this study was to determine if bifidobacteria could demonstrate potential antitoxin effects resulting from binding AFB_1. Enzyme-linked immunosorbent assays (ELISA) and [^{3}H]AFB_1 binding assays were used to quantitate AFB_1 binding activity for six different strains of bifidobacteria, two strains of *Escherichia coli*, one strain of *Staphylococcus aureus*, and one strain of *Lactobacillus rhamonsus*. The data suggest that all isolates tested bound significant quantities of AFB_1 ranging from 25% to nearly 60% of the added toxin and that there are reproducible strain differences in AFB_1 binding capacity.

MATERIALS AND METHODS

Strains and cultivation. *Bifidobacterium longum* JR20, *Bifidobacterium adolescentis* 14, and *Bifidobacterium* spp. JO3 and CH4 were recently isolated from the feces of healthy human subjects. *Bifidobacterium* spp. Bf6, JO3, CH4, and *Bifidobacterium bifidum* BGN4 *(4)* were reported previously *(20)*. Strains of bifidobacteria, *L. rhamnosus* strain GG (ATCC 53103), and *S. aureus* were cultured anaerobically in MRS broth (Difco, Detroit, Mich.) containing 5% (wt/vol) lactose. *E. coli* (Media Prep Lab, Michigan State University) and *E. coli* DH5α were cultured aerobically in Luria-Bertani broth. All strains were incubated at 37°C and shaken at 150 rpm until late log phase (optical density of 1.5). Cells were collected by centrifugation at 1,000 × *g* for 15 min at 4°C, washed twice with phosphate-buffered saline (PBS), followed by a final wash with distilled water. The cells were dried using a Speed-Vac Concentrator (Savant Instruments, Farmingdale, N.Y.) or a LYPH-

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LOCK 6 freeze dryer (Labconco, Kansas City, Mo.) and suspended in Hanks' buffered salt solution (Sigma, St. Louis, Mo.) to a concentration of 10 mg/ml. All bacterial cells were killed by heating at 95°C for 30 min. Cultures were stored frozen at −20°C until used.

AFB_1 binding assay: ELISA. Binding of AFB_1 by heat-killed bacterial cells was analyzed using modifications to the method of El-Nezami et al. *(9)*. Two milligrams of each bacterial strain in Hanks' buffered salts (10 mg/ml) were incubated with 500 ng AFB_1 in 50 μl PBS (10 μg/ml) for a final volume of 250 μl. As negative controls, PBS (pH 7.2) substituted for either the bacterial cells or AFB_1. The mixtures were incubated for 30 min at 37°C, with shaking at 150 rpm and then centrifuged at 2,000 × *g* for 15 min at 4°C. After centrifugation, 200 μl of supernatant was removed for ELISA analysis. The remainder of the supernatant was discarded and the cell pellet was suspended in 250 μl of PBS. The mixture was vortexed and centrifuged. Again, 200 μl of supernatant was removed and assayed (first wash). The cycle including addition of PBS, vortexing, centrifugation, and removal of supernatant was repeated five additional times (washes 2 through 6). Then, a chloroform wash was done. Each sample for the ELISA analysis was diluted 1:10 and 1:50 in PBS in triplicate. ELISA procedure was performed according to the previously published method by Pestka et al. *(21)*.

[^{3}H] AFB_1 binding assay. All bacterial strains were incubated with 20 μl of PBS containing 42 ng/ml [^{3}H]AFB_1 (Moravek Biochemicals, Brea, Calif.) plus 50 μl of PBS containing 500 ng AFB_1. PBS was again used as a substitute for either the bacterial cells or AFB_1 (negative controls). *L. rhamnosus* strain GG (LGG) was used as a positive control, previously found to bind 40% of total AFB_1 *(8)*. The same incubation conditions described above were followed. For an estimate of total AFB_1 added, two 20-μl aliquots were removed prior to beginning the washes and measured. The cell mixtures were then centrifuged at 2,000 × *g* for 5 min at 4°C. The six washes were performed as described above with a 5-min centrifugation time (only 20 μl was required for analysis of the [^{3}H]AFB_1 assay). For an approximation of AFB_1 remaining in each pellet following the washes, 270 μl of PBS was added. After vortexing, two 20-μl aliquots were removed. All analyses were done in triplicate. The 20-μl aliquots from above were added to 5 ml of High Flash Point Cocktail (Research Products International Corp., Mount Prospect, Ill.) in scintillation vials. The AFB_1 retrieved after each step was analyzed by the Minaxi Tri-Carb 4000 series scintillation counter for 2 min.

Statistical analysis. All 10 bacterial strains were compared utlilizing nonparametric statistics with no assumption of normality. The data set was first analyzed with a Kruskal-Wallis test (n = 30 with 9 degrees of freedom), resulting in a P-value of 0.021. This rank transformation test was followed by a second rank transformation test (Mann-Whitney U test) that found 20 of the 21 pairwise comparisons significantly different.

RESULTS AND DISCUSSION

The main objective of the study was to determine if different strains of bifidobacteria can bind AFB_1 in vitro. Aflatoxin binding to cells was defined as the sum of the [^{3}H] label contained in the six washes plus the label left in the cell pellet divided by the total AFB_1 added. Using this definition, strains of bifidobacteria were observed to have different binding affinities for AFB_1 (Fig. 1). LGG was used in this study as a benchmark (positive control) for our

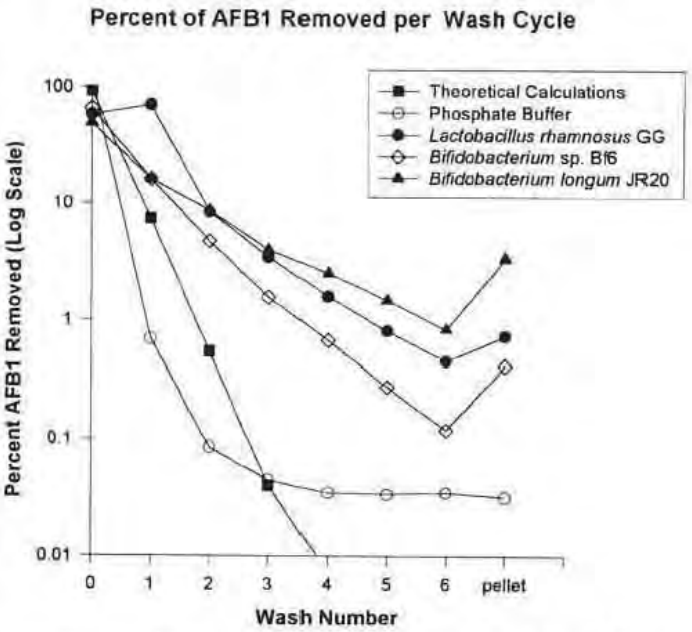

FIGURE 1. *AFB_1 binding affinity to different strains of* Bifidobacterium *and other bacteria. The binding affinity of different bacterial strains. Percent bound is the sum of aflatoxin retrieved in six washes plus the pellet, divided by the total amount of aflatoxin at the start of the reaction.*

methods in comparison to the high-performance liquid chromatographic method employed on this strain by El-Nezami et al. LGG was found to bind 37 ± 1% (Fig. 1), a value comparable to that found by El-Nezami et al. (40% binding of AFB_1 by heat-killed cells) *(9)*. The negative control (PBS substituted for cells) resulted in little retention of AFB_1 (Fig. 2). The second negative control (PBS substituted for AFB_1) resulted in a reading equivalent to the background reading of the scintillation counter (data not shown). The highest binder, *B. bifidum* BGN4, was found to bind 46 ± 4% of the total AFB_1 added to the cells. *Bifidobacterium* sp. JO3, *B. longum* JR20, and *Bifidobacterium.* sp. CH4 bound 41 ± 3%, 37 ± 3%, and 37 ± 1%, respectively. The final two strains of bifidobacteria, *B. adolescentis* 14 and *Bifidobacterium* sp. Bf6, bound 31 ± 3% and 25 ± 4%, respectively. The quantity of AFB_1 bound using ELISA analysis was consistent for five of the six bifidobacterial strains (data not shown). The only isolate not consistent was BGN4 that only bound 30% (via ELISA analysis performed in triplicate). The difference between ELISA and [^{3}H] methods for BGN4 could be explained by possible greater affinity of the [^{3}H]AFB_1 to the cell wall of BGN4 as compared to unmodified AFB_1. *Bifidobacterium* BGN4 was previously reported to show a very high cell surface hydrophobicity *(15)*. It might be interesting to examine if cell surface hydrophobicity is related to aflatoxin binding.

It has been speculated previously that cell wall composition may affect binding *(3, 9)*. Binding of AFB_1 by several other bacterial strains was therefore evaluated in order to determine if affinity was affected by the genus of gram-positive organism utilized and if gram-negative organisms have similar binding affinities (composition of the cell wall). *Staphylococcus aureus,* a nonenteric gram-posi-

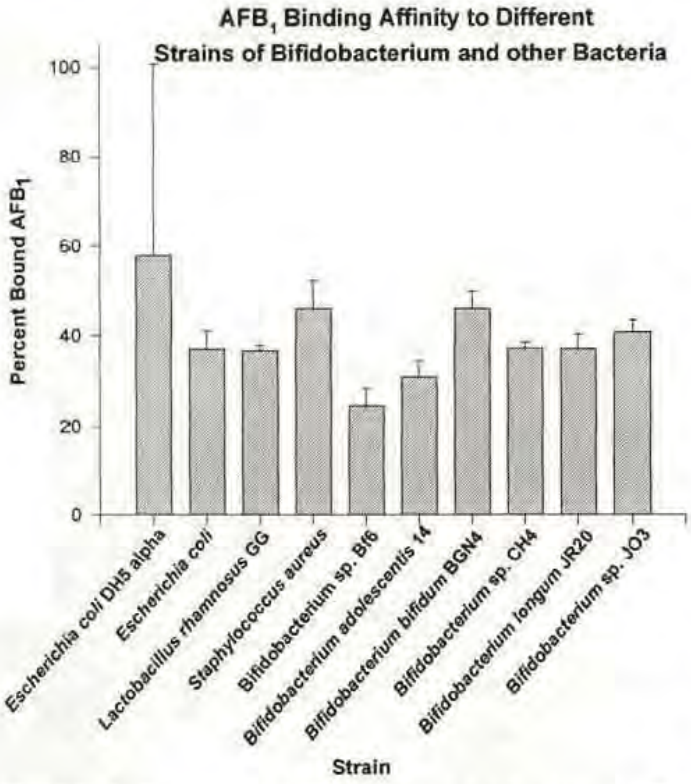

FIGURE 2. *Percentage of AFB_1 removed per wash cycle. The percentage of AFB_1 removed for Bf6, JR20, and the PBS positive control are compared to the theoretical calculations. Theoretical calculations were performed to assess the possibility of AFB_1 trapping in the bacterial pellet as the sole mechanism of AFB_1 removal. As shown, binding by Bf6 and JR20 was greater than the possible contribution by trapping. As a conservative estimate 7.4% of AFB_1 is trapped after each wash.*

tive bacterium commonly found in the nasal passage was found to bind 46 ± 6% of the total aflatoxin, equivalent to the percent bound by BGN4. Two *E. coli* strains, *E. coli* (Media Prep Lab, Michigan State University) and the laboratory strain DH5∝ served as test organisms to investigate this possibility. These strains bound 37 ± 4% and 58 ± 43%, respectively, placing them in the medium and high binder ranges compared to the bifidobacteria strains. The high standard deviation for DH5∝ was due to a possible outlier that bound 106% of the aflatoxin. Because the analyses were conducted in triplicate, the replicate could not be statistically eliminated as an outlier. Taken together these data suggest that enterics and gram-positive organisms bind aflatoxin with similar efficiency. These studies, however, cannot determine if the mechanisms of binding are similar or different.

The variations in binding between strains led us to believe that individual strains could carry multiple binding sites with different binding affinities. The washing steps described above were designed to investigate this possibility. If multiple binding sites with different affinities were present, a graph of wash number versus AFB_1 quantity in that wash should have rendered a nonlinear curve. Figure 2 shows that this was not the case for Bf6 and JR20. The linear slopes for Bf6 and JR20 suggest that, at least for these two strains, binding of AFB_1 occurred at sites with similar affinities, possibly identical sites with identical mechanisms (the remainder of the bifidobacterial strains showed the same result, data not shown). AFB_1 is removed from cells by washing at a relatively constant rate, therefore suggesting binding is not irreversible. These results contradict the findings of E. L. Nezami that found binding irreversible after 30 min sonication *(9)*.

The linear reduction of aflatoxin demonstrated in Figure 2 raised the question of whether aflatoxin was bound by the strains or if the toxin was merely being trapped in the bacterial pellet during centrifugation. To test the possibility of trapping, theoretical calculations were generated. The volume of the bacterial cell pellet after centrifugation was conservatively estimated to be 20 μl of the 270-μl total volume. The pellet then represents 7.4% of the total volume. If trapping was the sole mechanism for retention of AFB_1, only 7.4% of the AFB_1 would be retained in the pellet after each wash (Fig. 2). The slopes of the graphs of data sets for BF6 and JR20 are much shallower than the theoretical curve for trapping only. The data suggest that 4 to 5 logs more toxin remain bound to cells than would be predicted for trapping only. A negative control for trapping was performed where no cells (replaced by PBS) were added to the reaction. This control had a slope more similar to the theoretical calculation than to the bifidobacterial strains. The slope of LGG (positive control) was more similar to the slopes created by the data sets of BF6 and JR20. These data further demonstrate that retention of AFB_1 occurs by some mechanism of binding rather than trapping. Further research is required to determine the specific mechanism of binding and the effects the gastrointestinal tract may have on the binding ability of bifidobacteria.

ACKNOWLEDGMENTS

This work was partly supported by Research and Development Promotion Center for Ministry of Agriculture and Forestry, Korea (1999). The statistical analysis employed by Don Uzarski is gratefully appreciated.

REFERENCES

1. Ballongue, J. 1998. Bifidobacteria and probiotic action, p. 519–587. *In* S. Salminen and A. von Wright (ed.), Lactic acid bacteria microbiology and functional aspects. Marcel Dekker Inc., New York.
2. Bernet, M. F., D. Brassart, J. R. Neeser, and A. L. Servin. 1993. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. Appl. Environ. Microbiol. 59:4121–4128.
3. Ceigler, A., B. Lillehoj, R. E. Peterson, and H. H. Hall. 1966. Microbial detoxification of aflatoxin. Appl. Microbiol. 14:934–939.
4. Choi, Y. J., C. J. Kim, S. Y. Park, Y. T. Ko, H. K. Jeong, and G. E. Ji. 1996. Growth and b-glucosidase activity of *Bifidobacterium*. J. Microbiol. Biotechnol. 6:282–290.
5. Collins, E. B., and B. J. Hall. 1983. Growth of bifidobacteria in milk and preparation of *Bifidobacterium infantis* for a dietary adjunct. J. Diary Sci. 67:1376–1380.
6. Deguchi, Y., T. Morishita, and M. Mutai. 1985. Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. Agric. Biol. Chem. 49:13–19.
7. Ellis, W. O., J. P. Smith, B. K. Simpson, and J. H. Odham. 1991. Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control. CRC Crit. Rev. Food Sci. Nutr. 30:403–439.
8. El-Nezami, H., P. Kankaanpaa, S. Salminen, and J. Ahokas. 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B_1. Food Chem. Toxicol. 36:321–326.

9. El-Nezami, H., S. J. Salminen, and J. Ahokas. 1996. Biologic control of food carcinogens with use of *Lactobacillus* GG. Nutr. Today Suppl. 31:41S–42S.
10. Hoogkamp-Korstanje, J. A. A., J. G. E. M. Lindner, J. H. Marcelis, H. Den Daas-Slagt, and N. M. De Vos. 1979. Composition and ecology of the human intestinal flora. Antonie Van Leeuwenhoek 45:35–40.
11. Hughes, D. B., and D. G. Hoover. 1995. Viability and enzymatic activity of bifidobacteria in milk. J. Dairy Sci. 78:268–276.
12. Intizarov, M. M. 1979. Certain symbotic and antagonistic mechanism in bifidobacteria and their relationship to establishment of local immunity in gnotobiotic animals. Lenina 9:32–35.
13. Kim, H. S. 1988. Characterization of lactobacilli and bifidobacteria as applied to dietary adjuncts. Cult. Dairy Prod. J. 23:6–9.
14. Lankaputhra, W. E. V., and N. P. Shah. 1998. Antimutagenic properties of probiotic bacteria and of organic acids. Mutat. Res. 397: 169–182.
15. Lee, M. J., I. H. Kim, Z. Zhang, H. K. Shin, and G. E. Ji. 1998. Evaluation of human large intestinal bacteria and strain characterization of *Bifidobacterium*, p. 23–32. *In* Proceedings of the International Symposium on Probiotic Researches on Lactic Acid Bacteria, The Korean Society for Applied Microbiology, Seoul, Korea.
16. Line, J. E., and R. E. Brackett. 1995. Role of toxin concentration and second carbon source in microbial transformation of aflatoxin B_1 by *Flavobacterium aurantacium*. J. Food Prot. 58:1042–1044.
17. Massey, T. E., R. K. Stewart, J. M. Daniels, and L. Liu. 1995. Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B_1 carcinogenicity. Proc. Soc. Exp. Biol. Med. 208:213–227.
18. McLean, M., and M. F. Dutton. 1995. Cellular interactions and metabolism of aflatoxin: an update. Pharmacol. Ther. 65:163–192.
19. Modler, H. W., R. C. McKellar, and M. Yaguchi. 1990. Bifidobacteria and bifidogenic factors. Can. Inst. Food Sci. Technol. J. 23:29–41.
20. Park, S. Y., G. E. Ji, Y. T. Ko, H. K. Jung, Z. Ustunol, and J. J. Pestka. 1999. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. Int. J. Food Microbiol. 46:231–241.
21. Pestka, J. J., Y. Li, W. O. Harder, and F. S. Chu. 1981. Comparison of radioimmunoassay and enzyme-linked immunosorbent assay for determining aflatoxin M1 in milk. J. Assoc. Off. Anal. Chem. 64: 294–301.
22. Rotimi, V. O., and B. I. Duerden. 1981. The development of the bacterial flora in normal neonates. Med. Microbiol. 14:51–62.
23. Rustom, I. Y. S. 1997. Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. Food Chem. 59:57–67.
24. Thyagaraja, N., and A. Hosono. 1994. Binding properties of lactic bacteria from 'idly' towards food-borne mutagens. Food Chem. Toxicol. 32:805–809.

J. Microbiol. Biotechnol. (2002), **12**(3), 398–405

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cytoskeleton Reorganization and Cytokine Production of Macrophages by Bifidobacterial Cells and Cell-Free Extracts

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Received: November 15, 2001
Accepted: April 26, 2002

Abstract Bifidobacteria have been previously shown to stimulate the immune functions and cytokine production in macrophages and T-lymphocytes. Accordingly, the RAW 264.7 murine macrophage cell line was used to assess the effects of *Bifidobacterium* on the proliferation and cytoskeleton reorganization of the cells. Cytokine production after exposure to *Bifidobacterium* was also monitored in both whole cells and cell-free extracts. When RAW 264.7 cells were cultured for 24 h in the presence of heat-killed *Bifidobacterium bifidum* BGN4, the proliferation of macrophages was slowed down in a dose-dependent manner and cell differentiation was observed by staining with the actin-specific fluorescent dye, rhodamin-conjugated phalloidin. Although EL-4 cells, a T-cell line, stimulated RAW 264.7 cells to produce TNF-α and IL-6, the stimulatory activity of *B. bifidum* BGN4 decreased as the EL-4 cell mumber increased. When disrupted and fractionated BGN4 was used, the whole cell fraction was more effective than the other fractions for the TNF-α production. In contrast, the cell-free extract exhibited the highest IL-6 production level among the fractions, which was evident even at a 1 μg/ml concentration. The current results demonstrate that *Bifidobacterium* induced differentiation of the macrophages from the fast proliferative stage and that the cytokine production was differentially induced by the whole cells and cell-free extracts. The *in vitro* approaches employed herein are expected to be useful in further characterization of the effects of bifidobacteria with regards to gastrointestinal and systemic immunity.

Key words: *Bifidobacterium*, macrophage activation, cytoskeleton, actin

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An immunopotentiator has been defined as a substance that directly or indirectly enhances a particular immunological function. Immunopotentiators of bacterial origin occupy an important position in the field of immunology related to vaccination and immunotherapy. Many studies have already shown that pathogenic bacteria or their fractions stimulate cytokine production both *in vitro* and *in vivo* [9]. Although these bacteria can increase immune functions, they are undesirable in hosts. Strains of the genera *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* which are commonly referred to as lactic acid bacteria (LAB) are considered to be nonpathogenic and believed beneficial to human health [16]. In particular, bifidobacteria are excellent candidates for immunomodulation studies of the intestinal system, because they are widely used in commercial fermented dairy products and are one of the most abundant groups of microorganisms in the human gut, with 3–4 logs more cells per gram of feces than *Lactobacillus* [1]. It has recently been reported that bifidobacteria enhance several immune functions, namely macrophage and lymphocyte activation [8, 28], antibody production [12, 13, 36, 37], and a mitogenic response in spleen and Peyer's patches [9, 10, 12, 33]. Such stimulation of immune responses by bifidobacteria has been proposed to enhance resistance to infection by pathogenic organisms [4, 23, 35] and potentially prevent cancer [5, 21, 25, 26].

Macrophages play a major role in a host's defense against infection and tumor formation. It is believed that macrophages regulate immunity through the production of several mediators, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) [11], NO, and H_2O_2, thereby inhibiting tumor cells, bacteria, fungi, and parasites [14, 29]. While these mediators play key homeostatic functional roles, they are also potentially capable of injuring host

tissues [6]. Thus, the regulation of these mediadiators is important for maintaining a normal physiological immune state. *Bifidobacterium* can differentially upregulate the production of macrophage cytokines in a dose-dependent fashion. The exact mechanism for the induction of an immune response by *Bifidobacterium* is unclear because of the complexity of the immune reactions involving different interacting cells and poor characterization of the *Bifidobacterium*-derived components in the immune system. The cell components of *Bifidobacterium*, which function as immunomodifiers of the host, have been reported to include peptidoglycans, intra and extracellular polysaccharide products, cell-free extracts, and cell wall preparation [7, 8, 9, 17, 25, 26]. Since most previous studies have focused on the cell wall component for the immunopotentiators from *Bifidobacterium* [25, 26], little attention has been paid to other fractions. Furthermore, there is not as yet a clear understanding of the molecular and cellular basis for bifidobacteria immunostimulation.

In previous studies, by the current authors, various strains of bifidobacteria were compared for the macrophage activation [18]. The data revealed that high-activating strains tended to induce all the macrophage activating markers: TNF-α, IL-6, H_2O_2, NO, and a phagocytic ability, at higher level than low stimulating strains. Accordingly, further characterization of the effect of *Bifidobacterium* strain specificity on macrophages may provide more insight into the role of specific *Bifidobacterium* strains in the structure-functional aspects of macrophage activation.

The current study observed that the activation of macrophages by bifidobacteria was accompanied by slow proliferation and greater differentiation, especially through the action of filament formation. Also, TNF-α and IL-6 production was differentially induced by whole cells and cell-free extracts, respectively.

MATERIALS AND METHODS

Bifidobacterium Cultures

The identification and experimental use of *B. bifidum* BGN4 and *Bifidobacterium* sp. CN2 were previously reported [18]. All strains were cultured and subcultured anaerobically in an MRS broth (Difco, Detroit, MI, U.S.A.) containing 5% lactose (wt/vol, MRSL) at 37°C until the late log phase. The cells were collected by centrifugation at 1,000 ×g for 15 min at 4°C and washed twice with PBS, followed by a final washing with distilled water. The cells were then dried using a Speed-Vac (Speed-Vac Instruments, Inc., NY, U.S.A.) and resuspended with Hanks' buffered salt solution (Sigma Chemical Co., St. Louis, MO, U.S.A.) to the desired bacterial concentration on a dry weight basis. For introduction into a tissue culture, the bifidobacteria were heat-killed by heating at 95°C for 30 min. The heat-killed cultures were aliquoted and stored at −80°C until use.

Chemicals and Reagents

The TNF-α, IL-6, purified antibodies to TNF-α or IL-6 (rat anti-mouse), and biotinylated rat anti-mouse TNF-α or IL-6 were obtained from PharMingen (San Diego, CA, U.S.A.). The Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Chagrin Falls, IL, U.S.A.). The tetramethylbenzidine (TMB) was from Fluka Chemical Corp. (Ronkonkoma, NY, U.S.A.), and the MTT [3-(4,5-di-methylthizol-2-yl)-2,5-diphenyltetrazolium bromide] and Phalloidinrhodamine were from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Cell Culture

The mouse macrophage cell line RAW264.7 (American type culture collection) and mouse thymoma cell line EL-4 were grown in DMEM supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, streptomycin (100 μg/ml), and penicillin (100 U/ml). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. The cell number and viability were assessed by the trypan blue dye exclusion method [32] on a Neubauer hemacytometer (American Optical, Buffalo, NY, U.S.A.). The cells were grown to confluence in sterile tissue culture dishes and gently detached by repeated pipetting. For the experiments, the cells were cultured in triplicate at various densities in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, U.S.A.). To investigate the effect of co-culturing, macrophages, prepared as described above and pretreated with the culture medium alone or with various bacterial concentrations, were incubated together with EL-4 cells at a ratio of 1:0.3 to 1:10 for 24 h. After incubation, the culture supernatants were used for the TNF-α and IL-6 analysis.

TNF-α and IL-6 Quantitation

The production of TNF-α and IL-6 was monitored by ELISA (Enzyme Linked ImmunoSorbent Assay) using a modification of the procedure of Dong *et al.* [3]. Briefly, microtiter strip wells (Immunolon IV Removawell; Dynatech Laboratories, Chantilly, VG, U.S.A.) were coated overnight at 4°C with 50 μl of 1 μg/ml purified antibodies to TNF-α or IL-6 antibodies (rat anti-mouse) in a 0.1 M sodium bicarbonate buffer (pH 8.2). The wells were then incubated with 300 μl of 3% (v/v) bovine serum albumin (BSA) in 0.01 M PBS (pH 7.2) containing 0.2% (v/v) Tween 20 (PBST) at 37°C for 30 min to block any nonspecific protein binding. Next, standard recombinant murine TNF-α, IL-6, and the samples were diluted in 10% (v/v) FBS RPMI-1640, and 50 μl aliquots were added to appropriate wells, and incubated at 37°C for 1 h. After washing four times

with PBST, the biotinylated rat anti-mouse TNF-α or IL-6 antibodies were diluted in BSA-PBST to 1 μg/ml and 1.5 μg/ml, respectively, and a further 50 μl of these were added and the mixture was incubated at room temperature for 1 h. The plates were washed six times and incubated with 50 μl of a streptavidin-horseradish peroxidase conjugate (1.5 μg/ml in BSA-PBST) at room temperature for 1 h. After washing eight times, the bound peroxidase conjugate was detected by adding a 100 μl/well solution of a substrate consisting of 25 ml of a 0.1 M citric-phosphate buffer (pH 5.5), 0.1 mg/ml TMB, and 100 μl of 1% H_2O_2. An equal volume of 6 N H_2SO_4 was then added to stop the reaction. The plates were read at 450 nm on a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA, U.S.A.). The TNF-α and IL-6 were quantitated using V_{max} Software (Molecular Devices).

MTT Assay

A colorimetric MTT cleavage test was performed as described by Visconti *et al.* [34], to assess the proliferation in the RAW 264.7 cultures. Briefly, 50 μl of a filter-sterilized stock MTT solution (5 mg/ml in phosphate buffered saline) was added to each well, then the cultures were incubated for 3 h at 37°C. The plates were centrifuged at 450 ×g and the supernatant removed. Isopropanol (200 μl) was added to the wells and mixed thoroughly to completely dissolve the crystalline material. The absorbance of each tissue culture well was read on a V_{max} Kinetic Microplate Reader at 570–690 nm.

Lysosome Staining with Acridine Orange

To locate the macrophage lysosomes, the RAW 264.7 cells exposed to *Bifidobacterium* were incubated with DMEM containing acridine orange (10 μg/ml) for 10 min, then the excess fluorescence dye was removed by washing twice with DMEM without the dye at 5-min intervals for 5 min [38]. The cells were observed to be alive with an Olympus epifluorescence microscope (DX-05F-3, Olympus Co., Japan) and photographed using TMAX 400 film (Kodak, Rochester, NY, U.S.A.)

Actin Filament Staining

An actin filament was visualized by staining with phalloidin-rhodamine. The cells treated with bifidobacteria were fixed with 3% paraformaldehyde for 10 min and washed 3 times with phosphate-buffered saline (PBS). For staining, the cells were incubated in PBS containing 0.1 μg/ml phalloidin-rhodamine for 10 min and washed extensively with PBS. The cells were mounted on a glass slide and observed under an Olympus epifluorescence microscope.

Preparation of *Bifidobacterium* BGN4 Cell Fractions

The cells were fractionated by a modification of the method of Okitsu-Negishi *et al.* [18]. The cells grown in the MRS medium were pelleted by centrifugation (1,000 ×g for 20 min). The pellets were then washed twice with PBS and centrifuged again. The packed cells were suspended homogeneously in 30 ml of distilled water, and then disintegrated using a French Press (Spectronic, Rochester, NY, U.S.A.). The whole cells and debris were removed by centrifugation at 3,000 ×g for 10 min at 4°C. The cell walls were sedimented by centrifugation at 10,000 ×g for 30 min at 4°C, and the supernatant used as the cell-free extract. The crude-wall fractions were checked microscopically. The cell walls were washed another two times in 15 mM NaCl, followed by a wash in 50 mM TrisHCl, and then diluted in twice the volume of 10 mM potassium phosphate buffer (pH 7.0). RNase and DNase were added to a final concentration of 50 μg/ml. The mixture was incubated at 37°C for 90 min and then centrifuged at 10,000 ×g for 40 min. The washed cell walls were treated with 2% sodium dodecyl sulfate (SDS) and heated at 70°C for 2 h to remove the membrane. The cell walls were then washed extensively with distilled water to remove the SDS, collected by centrifugation, and lyophilized for use as the purified cell-wall preparation.

Statistical Analysis

The data were analyzed by the Student-Newman-Keuls (SNK) test following a one-way analysis of variance (ANOVA) using the Sigmastat Statistical Analysis System (Jandel Scientific, San Rafael, CA, U.S.A.). A probability of $p<0.05$ was used in the two-tailed test as the criterion for statistical significance.

Results

Effect of *Bifidobacterium* on Macrophage Cytoskeleton Organization

The current authors previously demonstrated that both human and commercial *Bifidobacterium* strains can stimulate H_2O_2, NO, TNF-α, and IL-6 from the macrophage cell line, and this effect is strain-specific and dose-dependent [18]. Among the 33 *Bifidobacterium* strains tested, *B. bifidum* BGN4 showed the highest stimulation effect for cytokine production even at a low concentration of cells (10 μg/ml), whereas strain CN2 showed the lowest activity. To further examine the morphological change at a subcellular level during the activation of the macrophages by bifidobacteria, the staining of a lysosome-like vacuole and actin filament were performed. Lysosome-like vacuole staining is based on the use of lysosomotropic weak-base acridine orange, which in its stacked form as it occurs within lysosomes, emits red fluorescence when excited by blue light. After treatment with the *Bifidobacterium* CN2 strain which exhibited a lower potency for macrophage activation, the macrophages showed a slight change in the red fluorescence intensity of the small vacuoles (Fig. 1Ab). However, when

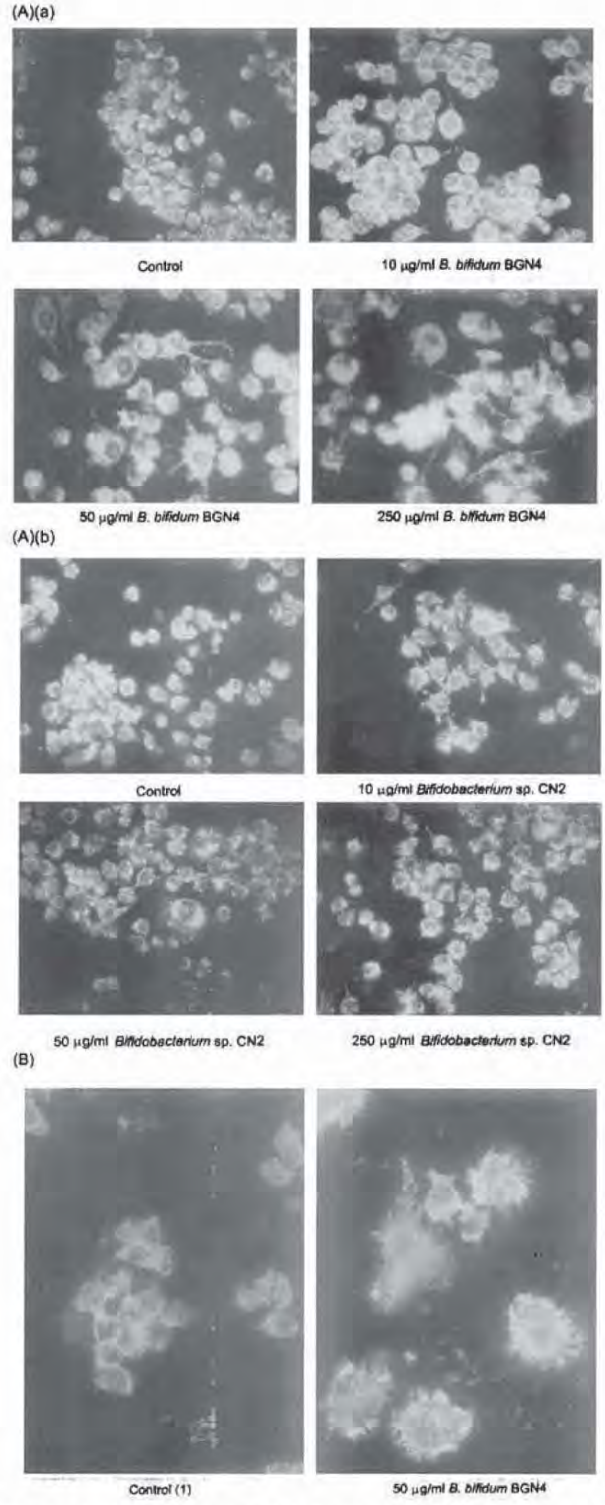

Fig. 1. Acridine orange staining (A) and actin filament staining (B) of murine macrophage RAW 264.7 cells treated with *Bifidobacterium* cells.

A: Macrophage cells treated with the high-macrophage activating strain BGN4 (a) showed an increased number of activated vacuoles compared to those treated with the low-macrophage activating strain CN2 (b). B: The *B. bifidum* BGN4-treated macrophage cells showed a much greater level of actin filament formation and filopodia development (magnification ×400).

treated with the *B. bifidum* BGN4 strain, the cells showed greater numbers and larger sizes of activated vacuoles with a significantly increased red fluorescence intensity. This increase was markedly potentiated when increasing the cell concentration of *B. bifidum* BGN4 (Fig. 1Aa). Besides the activation of the lysosome-like vacuoles, the exposure of the macrophages to *B. bifidum* BGN4 enlarged the size of the macrophage cells 3–4 times. Since the altered macrophage morphology could be contributed to a change in the microfilament arrangement, the organization of the actin filaments was determined. Phalloidin, a toxin from the plant *Amanita phalloides*, irreversibly polymerizes actin filaments, and thus can recognize filamentous actin. The changes in the cell shape and in the patterns of the microfilament distribution were analyzed using cell monolayers before and after exposure to the bacteria. A few minutes after the addition of the bifidobacteria, the staining of the F-actin with rhodamine phalloidin revealed the formation of large numbers of filopodia around the periphery of the cells (Fig. 1B). A simultaneous increase in the fluorescence intensity also occurred in response to the bifidobacteria. The MTT cleavage test has previously been used as an indirect measure of the proliferation of cultured cells [34]. As the concentration of *Bifidobacterium* BGN4 increased from 0 to 50 μg/ml the MTT value decreased in a dose-dependent manner (Fig. 2). This result suggests that, upon activation of the macrophages by the *Bifidobacterium*, the proliferation activity of the macrophages decreased.

Effect of *Bifidobacterium* on Cytokine Production in Co-Culture System of Macrophage and T-Cell Lines

To examine the effect of the *Bifidobacterium* on cytokine production in a system more resembling an *in vivo* situation, T-cells were added to the assay. The co-culture system of macrophages and T-cell lines was considered to reflect *in vivo* conditions better than a single cell line culture. For the experiment, RAW 264.7 cells (5×10^5 cells/ml) were co-incubated with an increasing ratio of EL.4

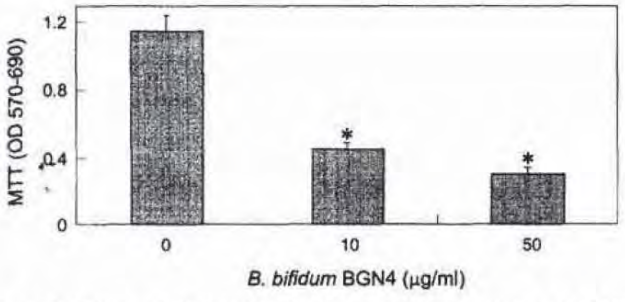

Fig. 2. Effect of *B. bifidum* BGN4 on MTT activity of RAW 264.7 cells (5×10^5 cells/ml).

The cells were incubated in the presence of different cell concentrations of *Bifidobacterium* for 24 h. Data are means±SD of triplicate cultures. Asterisk indicates significant difference from control (*B. bifidum* BGN4, 0 cells/ml) ($P<0.05$).

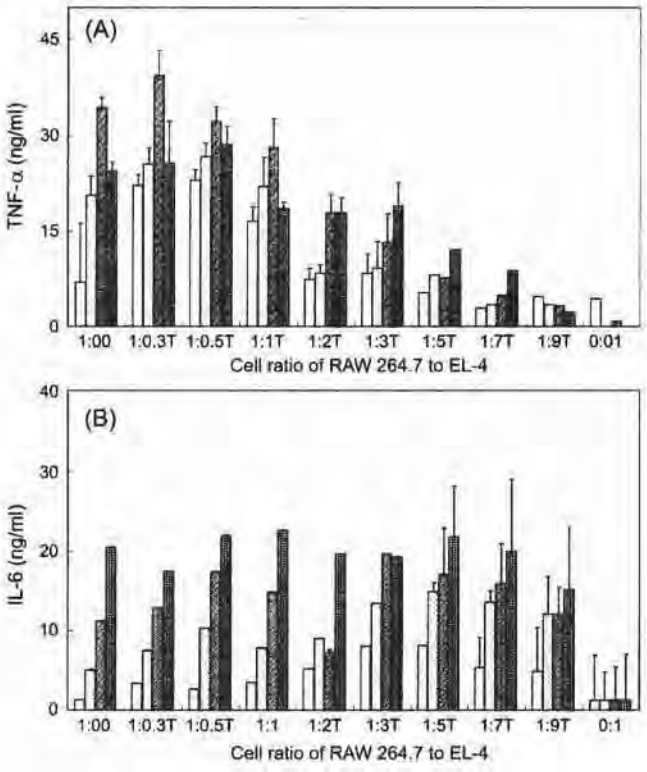

Fig. 3. Effect of *B. bifidum* BGN4 cells on the production of TNF-α (A) and IL-6 (B) by co-cultured RAW 264.7 and EL-4 cells.

Data are means±SD of triplicate cultures. Symbols: □ 0, ▒ 10, ▨ 50, ▦ 250 μg/ml *B. bifidum* BGN4.

IL-2 thymoma cells (T-helper cell model) (Fig. 3) from 1:0 to 1:9. In the absence of the *Bifidobacterium*, the production of cytokines gradually increased up to an 1:0.5 (RAW 264.7: EL-4) ratio for TNF and up to a 1:5 ratio for IL-6, followed by a gradual decrease as the EL-4 concentration increased further. These results show that the EL-4 cells were able to influence the production of cytokine from the RAW 264.7 cells. Although the EL-4 cells stimulated the RAW 264.7 cells to produce TNF-α and IL-6, the stimulatory activity of the *Bifidobacterium* decreased, especially with regard to the production of TNF-α, as the EL-4 concentration increased, although *B. bifidum* BGN4 alone increased the production of TNF-α and IL-6.

Effect of Cell Fractions of *B. bifidum* BGN4 on TNF-α and IL-6 Production

To examine the effect of cell fractions of *B. bifidum* BGN4 on TNF-α and IL-6 production from macrophages, RAW 264.7 cells were incubated with 0–250 μg/ml of bacterial fractions, and the cytokine secretion in the culture supernatant was monitored by ELISA. The results for TNF-α and IL-6 production are shown in Figs. 4A and 4B, respectively. While the whole cell fraction was more effective for TNF-α production than the other fractions, the cell-free extracts exhibited the strongest IL-6 production, followed by the purified cell wall and whole cells. The cell-free extract produced IL-6 even at 1 μg/ml concentration.

DISCUSSION

Bifidobacteria and other lactic acid bacteria have been previously shown to stimulate immune functions [7, 10, 12, 19] and antitumor activity in a host [5, 21, 25, 27]. Thus, their ability to stimulate macrophages and T-cells [8, 26, 27] may play a crucial role in these activities. Macrophages are known to facilitate the presentation of various antigens to lymphocytes and provide signals in the form of cell-to-cell and humoral-to-cell interactions that result in an enhanced lymphocyte proliferation and elaboration of various cytokines [11]. Therefore, cytokine production is likely to be a good indicator of the degree of macrophage activation. Previously, we showed that both human and commercial *Bifidobacterium* strains could stimulate H_2O_2, NO, TNF-α, and IL-6 production and this effect was strain dependent. These results were consistent with previous reports that bifidobacteria enhance

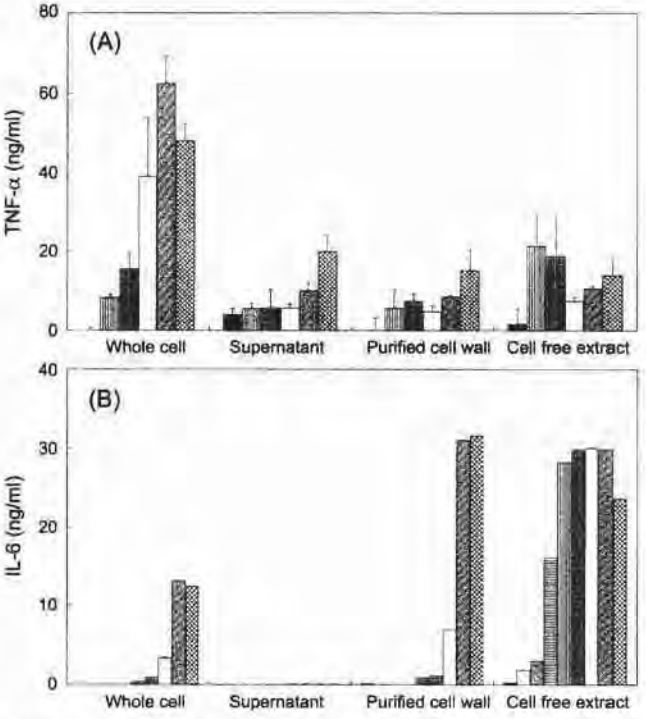

Fig. 4. Effect of various cell fractions of *B. bifidum* BGN4 on the production of TNF-α (A) and IL-6 (B) by murine macrophage cell lines.

RAW 264.7 cells (5×10^5 cells/ml) were cultured for 24 h in the presence of various bacterial components. Data are means±SD of triplicate cultures. Symbols: ■ 0, ▒ 0.1, ▧ 0.25, ▤ 0.5, ▥ 1, ▦ 5, □ 10, ▨ 50, ▩ 250 μg/ml *B. bifidum* BGN4.

the production of TNF-α and IL-6 by human peripheral blood mononuclear cells [16, 30] and mouse peritoneal cells [27]. Lee *et al.* [12] reported that bifidobacteria significantly increased IL-2 production in EL-4 cells with PMA stimulation. However, the results were in contrast with Solis Pereyra and Lemonnier [30] who were unable to detect any increase of IL-2 production during the incubation of blood mononuclear cells with the *Bifidobacterium* sp. used in dairy production. The co-culture system of macrophages and T-cell lines employed in the current study appeared to reflect *in vivo* conditions better than a single cell line culture. In the control groups, either RAW 264.7 cells or EL-4 cells were cultured with *B. bifidum* BGN4 to measure the production of BGN4-stimulated TNF-α and IL-6. No production of the macrophage cytokine TNF-α and IL-6 was detected or minimally detected in the EL-4 thymoma cell line, when incubated with *Bfidobacterium* (data not shown). Marin *et al.* [15] also reported that *Bifidobacterium* had negligible effects on interleukin production in a culture of EL-4 cells without PMA stimulation. The current results showed that the co-incubation of the RAW 264.7 cells with *B. bifidum* BGN4 or EL-4 cells enhanced the production of both TNF-α and IL-6 from the RAW 264.7 cells, yet there was no synergistic effect between the *Bifidobacterium* and the T-cells. Perhaps, the EL-4 cells compete with the *Bifidobacterium* in the activation of RAW 264.7, thereby reducing the stimulatory effect of the *Bifidobacterium* on the RAW 264.7 activation.

The development of stress fibers may be related to an altered cellular morphology in the macrophages. These changes probably result from the rearrangement of cellular cytoskeletons and extracellular matrix components. Actin is one such cellular component that has already been identified to be involved in the cellular process. The current study also provides evidence to relate stress fiber formation with the enlargement of the macrophage cell size and increased fluorescence intensity. This highly developed actin may aid the activated macrophage to migrate toward the target site with a higher chemotactic activity. In addition to actin, myosin also plays an important role in several cellular responses including contractility [31], thus the filamentous myosin structure may play a role in the *Bifidobacterium*-induced cell-shape change. It would be interesting to see if *Bifidobacterium* induces the rearrangement of myosin in the macrophages. Furthermore, with highly developed vacuoles, they may ingest pathogenic organisms more vigorously. In fact, the BGN-4 activated macrophages exhibited an enhanced phagocytic activity when challenged with fluorescein-labeled *E. coli* (unpublished results). Taken together, the present findings that a high-stimulating strain induced more marked development of actin filament and filopodia formation support the above-mentioned results related to the cytokine production.

The major site for immune responses in the intestinal tract is considered to be Peyer's patches, which are distributed differently depending on both age and species. Human Peyer's patches within the duodenum are small and consist of a few lymphocyte follicles, yet become larger in more distal areas in the ileum; as such, they are considered to be induced by the presence of endogenous microflora. The survival of a cell and processing of its cell component may also be related to the efficiency of the immunostimulatory effect. The death of *Bifidobacterium* may occur rapidly once they enter the immune system, due to their strict anaerobic characteristic. The ease of cell death and lysis is also related to structural integrity, composition of the cell material, and sensitivity to enzymatic or chemical attack. In this context, the binding of an intact cell or cell component may play a role in the immune activation with mechanisms different from each other. Indeed, it was noticed that whole cells stimulated TNF-α production, while cell-free extracts favored IL-6 production. In particular, the cell-free extract fraction exhibited IL-6 production even at a 1 μg/ml concentration. It is very interesting that the stimulative fractions for TNF-α or IL-6 were different in the *B. bifidum* BGN4 cell fractions. Lee *et al.* [12] reported that disrupted cells of *B. adolescentis* M101-4 enhanced the *in vitro* proliferation and secretion of antibodies specific for the nominal antigen, suggesting the polyclonal activation of B-cells and/or antibody-producing plasma cells. Yasui *et al.* [37] reported that *Bifidobacterium* stimulated IgA production in the gut. IL-6, secreted by macrophages, makes a critical contribution to the development of mucosal IgA responses. IL-6 drives IgA production when it is added to Peyer's patch B-cell cultures [2]. The increased production of macrophage IL-6 by the cell-free extract from *B. bifidum* BGN4 observed in the current study suggests that this may affect the IgA production in the B-cells. In contrast, Hatcher and Lambrecht [8] reported that extracts from *Bifidobacterium* produced activation in the absence of either viable organisms or cell wall fractions. An attempt was made to further purify the IL-6 stimulating components by Sephdex G-200 gel filtration chromatography, and DEAE- and Mono Q- ion-exchange chromatography (data not shown). Various fractions containing proteins or carbohydrates showed IL-6 stimulating activities. Some peaks containing carbohydrates without proteins also showed activity. The highly active fractions consisted of both carbohydrates and proteins. Hosono *et al.* [9] reported that three quarters of the dry weight of the isolated immunoactive fraction from *B. adolescentis* M101-4 consisted of carbohydrates, leading them to suggest that proteins did not seem to be directly related to the immunopotentiating activity, whereas polysaccharides were. Gomez *et al.* [7] reported that exocellular products from *Bifidobacterium*, which were carbohydrate-rich fractions, stimulated DNA synthesis in LPS-sensitive B-lymphocytes.

The current results suggest that both the protein components and the carbohydrates from the cell-free extract were important in enhancing IL-6 production. In conclusion, the results reported here suggest that the activation of macrophages was accompanied by actin filament formation and vacuole development. In addition, this stimulatory capacity was also affected by the dose, strain, and composition of the *Bifidobacterium*. The *in vitro* approaches employed here should be useful in future mechanistic characterization of the effects of bifidobacteria on gastrointestinal immunity and the possibility of enhancing gastrointestinal immune functions. Further elucidation of the differential effects of the cell-free extracts on cytokine production would contribute to a better understanding of the role of the *Bifidobacterium* in the intestinal immune system.

Acknowledgments

This work was supported by a research grant from the Korean Ministry of Science and Technology. The Authors also acknowledge the Department of Education, Korea.

References

1. Ballongue, J. 1998. Bifidobacteria and probiotic action, pp. 519–587. *In* Salminen S. and von Wright A. (eds.), *Lactic Acid Bacteria Microbiology and Functional Aspects.* Marcel Dekker Inc., New York, U.S.A.
2. Beagley, K. W., J. H. Eldridge., W. K. Aicher, J. Mestecky, S. Di Fabio, H. Kiyono, and J. R. McGhee. 1991. Peyer's patch B cells with memory cell characteristics undergo terminal differentiation within 24 hours in response to interleukin-6. *Cytokine* **3:** 107–116.
3. Dong, W., J. I. Azcona-Olivera, K. H. Brooks, J. E. Linz, and J. J. Pestka. 1994. Elevated gene expression and production of interleukins 2, 4, 5, and 6 during exposure to vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. *Toxicol. Appl. Pharmacol.* **127:** 282–290.
4. Duffy, L. C., M. A. Zielezny, M. Riepenhoff-Talty, D. Dryja, S. Sayahtaheri-Altaie, E. Griffiths, D. Ruffin, H. Barrett, J. Rossman, and P. L. Ogra. 1994. Effectiveness of *Bifidobacterium bifidum* in mediating the clinical course of murine rotavirus diarrhea. *Pediatr. Res.* **35:** 690–695.
5. Fernandes, C. F. and K. M. Shahani. 1990. Anticarcinogenic and immunological properties of dietary lactobacilli. *J. Food Prot.* **53:** 704–710.
6. Fukuo, K., T. Inoue, S. Morimoto, T. Nakahashi, O. Yasuda, S. Kitano, R. Sasada, and T. Ogihara. 1995. Nitric oxide mediates cytotoxicity and basic fibroblast growth factor release in cultured vascular smooth muscle cells. A possible mechanism of neo vascularization in atherosclerotic plaques. *J. Clin. Invest.* **95:** 669–676.
7. Gomez, E., M. M. Melgar, G. P. Silva, A. Portoles, and I. Gil. 1988. Exocellular products from *Bifidobacterium adolescentis* as immunomodifiers in the lymphoproliferative responses of mouse splenocytes. *FEMS Microbiol. Lett.* **56:** 47–52.
8. Hatcher, G. E. and R. S. Lambrecht. 1993. Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid-producing bacteria. *J. Dairy Sci.* **76:** 2485–2492.
9. Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S. Kaminogawa. 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M 101-4. *Biosci. Biotech. Biochem.* **61:** 312–316.
10. Kado-Oka, Y., S. Fujiwara, and T. Hirota. 1991. Effects of bifidobacteria cells on mitogenic response of splenocytes and several functions of phagocytes. *Milchwissenshaft* **46:** 626–630.
11. Laskin, D. L. and J. Pendino. 1995. Macrophages and inflammatory mediators in tissue injury. *Annu. Rev. Pharmacol. Toxicol.* **35:** 655–677.
12. Lee, J., A. Ametani, A. Enomoto, Y. Sato, H. Motoshima, F. Ike, and S. Kaminogawa. 1993. Screening for the immunopotentiating activity of food microorganisms and enhancement of the immune response by *Bifidobacterium adolescentis* M101-4. *Biosci. Biotech. Biochem.* **57:** 2127–2132.
13. Link-Amster, H., F. Rochat, K. Y. Saudan, O. Mignot, and J. M. Aeschlimann. 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol. Med. Microbiol.* **10:** 55–64.
14. Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell. 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. *J. Biol. Chem.* **268:** 1908–1913.
15. Marin, M. L., J. H. Lee, J. Murtha, Z. Stunol, and J. J. Pestka. 1997. Differential cytokine production in clonal macrophage and T-cell lines cultured with bifidobacteria. *J. Dairy Sci.* **80:** 2713–2720.
16. Miettinen, M., J. Vuopio-Varkila, and K. Varkila. 1996. Production of human necrosis factor alpha, interleukin-6 and interleukin-10 is induced by lactic acid bacteria. *Infect. Immun.* **64:** 5403–5405.
17. Namioka, S. 1985. Immunoresponsiveness of newborn piglets and peptidoglycan derived from *Bifidobacterium*. *Bifidobact. Microfl.* **4:** 3–14.
18. Okitsu-Negishi, S., I. Nakano, K. Suzuki, S. Hashira, T. Abe, and K. Yoshino. 1996. The induction of cardioangitis by *Lactobacillus casei* cell wall in mice. I. The cytokine production from murine macrophages by *Lactobacillus casei* cell wall extract. *Clin. Immunol. Immunopathol.* **78:** 30–40.
19. Om, A. S., S. Y. Park, and G. E. Ji. 1999. Comparison of nitric oxide, hydrogen peroxide and cytokine production in RAW 264.7 cells by *Bifidobacterium* and other intestinal bacteria. *J. Microbiol. Biotechnol.* **9:** 98–105.
20. Park, S. Y., G. E. Ji, Y. T. Ko, H. K. Jung, Z. Ustunol, and J. J. Pestka. 1999. Potentiation of hydrogen peroxide, nitric

oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. *Int. J. Food Microbiol.* **46:** 231–241.
21. Rafter, J. J. 1995. The role of lactic acid bacteria in colon cancer prevention. *Scand. J. Gastroenterol.* **30:** 497–502.
22. Sarih, M., V. Souvannavong, and A. Adam. 1993. Nitric oxide synthase induces macrophage death by apoptosis. *Biochem. Biophys. Res. Commun.* **191:** 503–508.
23. Sasaki, T., S. Fukami, and S. Namioka. 1994. Enhanced resistance of mice to *Escherichia coli* infection induced by administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *J. Vet. Med. Sci.* **53:** 433–437.
24. Schmidt, H. H. H. W. and U. Walter. 1994. NO at work. *Cell* **78:** 919–925.
25. Sekine, K., J. Ohta, M. Onishi, T. Tatsuki, Y. Shimokawa, T. Toida, T. Kawashima, and Y. Hashimoto. 1995. Analysis of antitumor properties of effector cells stimulated with a cell wall preparation (WPG) of *Bifidobacterium infantis*. *Biol. Pharm. Bull.* **18:** 148–153.
26. Sekine, K., E. Watanabe-Sekine, J. Ohta, T. Toida, T. Tatsuki, T. Kawashima, and Y. Hashimoto. 1994 a. Induction and activation of tumoricidal cells *in vivo* and *in vitro* by the bacterial cell wall of *Bifidobacterium infantis*. *Bifidobact. Microfl.* **13:** 65–77.
27. Sekine, K., E. Watanabe-Sekine, T. Toida, T. Kasashima, T. Kataoka, and Y. Hashimoto. 1994b. Adjuvant activity of the cell wall of *Bifidobacterium infantis* for *in vivo* immune responses in mice. *Immunopharmacol. Immunotoxicol.* **16:** 589–609.
28. Sekine, K., T. Kasashima, and Y. Hashimoto. 1994c. Comparison of the TNF-α levels induced by human-derived *Bifidobacterium longum* and rat-derived *Bifidobacterium animalis* in mouse peritoneal cells. *Bifidobact. Microfl.* **13:** 79–89.
29. Snyder, S. H. and D. S. Bredt. 1992. Biological roles of nitric oxide. *Sci. Amer.* **266:** 68–77.
30. Solis Pereyra, B. and D. Lemonnier. 1993. Induction of human cytokines by bacteria used in dairy foods. *Nutr. Res.* **13:** 1127–1140.
31. Somlyo, A. and A. Somlyo. 1994. Signal transduction and regulation in smooth muscle. *Nature* **372:** 231–236.
32. Strober, W. 1991. Trypan blue exclusion test for cell viability, pp, A.3.3–4. *In* Coligan, J. E., Kruisbeek, A. M., Marguiles, D. H., Shevach, E. M., and Strober, W. (eds.), *Current Protocols in Immunology*, Greene Pub. and Wiley-Interscience, New York, U.S.A.
33. Takahashi, T., T. Oka, H. Iwana, T. Kuwata, and Y. Yamamoto. 1993. Immune response of mice to orally administered lactic acid bacteria. *Biosci. Biotech. Biochem.* **57:** 1557–1560.
34. Visconti, A., F. Minervini, L. G. Lucivero, and V. Gambatesa. 1991. Cytotoxic and immunotoxic effects of *Fusarium* mycotoxins using a rapid colorimetric bioassay. *Mycopathologia* **113:** 181–186.
35. Yasui, H., J. Kiyoshima, and H. Ushijima. 1995. Passive protection against rotavirus-I induced diarrhea of mouse pups born to and nursed by dams fed *Bifidobacterium breve* YIT 4064. *J. Inf. Dis.* **172:** 403–409.
36. Yasui, H. and M. Ohwaki. 1991. Enhancement of immune response in Peyer's patch cells cultured with *Bifidobacterium breve*. *J. Dairy Sci.* **74:** 1187–1195.
37. Yasui, H., N. Nagaoka, A. Mike, K. Hayakawa, and M. Ohwaki. 1992. Detection of *Bifidobacterium* strains that induce large quantities of IgA. *Microbial Ecol. Health Dis.* **5:** 155–162.
38. Young, M. R., A. H. Gordon, and P. H. Hart. 1990. Tubular lysosomes and their drug reactivity in cultured resident macrophages and in cell-free medium. *Exp. Cell Res.* **190:** 283–289.

J. Microbiol. Biotechnol. (2003), 13(2), 276–281

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of Adhesion of *Bifidobacterium* sp. BGN4 to Human Enterocyte-Like Caco-2 Cells

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Received: October 14, 2002
Accepted: January 9, 2003

Abstract The adhesion of probiotic bacteria to the intestinal mucosa is one of the desirable properties for their colonization in the intestinal tract, where these bacteria constantly compete with other bacteria. The adhesion of different strains of bifidobacteria to Caco-2 cells was compared. Among the strains examined, BGN-4 showed the highest adhesion level and the greatest cell surface hydrophobicity (CSH). No close relationship was found between the adhesion and CSH of the strains. Upon protease and heat treatment, the adhesion of the BGN-4 to the Caco-2 cells decreased significantly. The cells grown at 42°C showed a lower CSH and self-aggregation levels than cells grown at 37°C. The treatment of EGTA did not have any effect on the adhesion. The degree of adhesion did not differ among the experimental groups in which galactose, mannose, or fucose were added in the adhesion assay mixture. The results suggest that the adhesion of the *Bifidobacterium* to the epithelial cells may be affected by the composition and structure of the cell membrane and interacting surfaces.

Key words: Adhesion, *Bifidobacterium*, Caco-2

Bifidobacteria were first discovered in the feces of infants by Tissier [20], who used the name *Bacillus bifidus* communis. Bifidobacteria are generally characterized as Gram positive, nonspore-forming, nonmotile, strictly anaerobes, and V- or Y-shaped bacteria. The G+C content of DNA varies from 55 to 67 mole%. They are saccharoclastic organisms, producing acetic and lactic acids in the molar ratio of 3:2, without CO_2 production except in the degradation of gluconate.

Bifidobacteria are important constituents of the normal intestinal microflora in both humans and animals [2, 21], with various beneficial probiotic effects on the well-being of the host [1, 3, 10]. Also, humans suffering from lactose malabsorption can possibly benefit from reduced lactose contents by the action of *Bifidobacterium* β-galactosidase [15, 19]. Recent studies suggest that *Bifidobacterium* may play a role in the reinforcement of immune functions and improved resistance to cancer [12, 20]. Because of the general belief that bifidobacteria are beneficial to the health of the host in both infants and adults, continuous efforts have been made to improve *Bifidobacterium* strains with enhanced probiotic effect and growth yield during the fermentation process.

For an appropriate application of bifidobacteria in the industry, strains are desired to satisfy several prerequisites. Strains possessing resistance to acid and bile salt are desired. Acid- and bile-resistant bifidobacteria are more likely to survive when they are exposed to gastric low pH and intestinal bile salt. Additionally, the adhesion ability to the intestinal mucosa is one of the desirable properties that have to be selected for their specific use in commercial preparations. Adhesion of *Bifidobacterium* strains to the colon surface may occur by an association of the bacteria with a secreted mucus gel or by adherence to the underlying epithelium [8]. Generally, adhesion can be ascribed to the interplay of attractive and repulsive forces between the approaching surfaces [9, 11]. However, the investigation of adhesion of the *Bifidobacterium* on intestinal mucosa has been scarce, and the exact mechanism of the adhesion has not yet been delineated [6].

The aim of this study was to compare the adhesion to Caco-2 cells by different strains of bifidobacteria isolated from the feces of Korean people, and characterization of the adhesion property. In order to gain insight into the structural properties of the surface of the strains, the relationship between bacterial cell surface hydrophobicity (CSH) and adhesion ability to Caco-2 cells was determined for each strain. By using the highest adhering strain, BGN-4, which also possessed the greatest CSH, the adhesive property was characterized.

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MATERIALS AND METHODS

Bacterial Strains

The bifidobacterial strains used in this study were mostly isolated from the feces of the Korean people by following the method of Mitsuoka [13] and information derived from earlier reports [16]. All of the strains were grown for 24 h at 37°C under anaerobic condition in the MRS broth (Difco, Michigan, U.S.A.) containing 0.05% (w/v) L-cysteine·HCl. Viable cell counts were estimated by a serial dilution process, and 100 µl of each diluted mixture were spread onto BL agar medium (Difco) with 5% horse blood. Then, the plate was incubated anaerobically for 2-3 days at 37°C.

Caco-2 Cell Culture

Caco-2 cells were used throughout this study. The Caco-2 cell line (ATCC, HTB 37) was originally isolated from a human colon adenocarcinoma and obtained from the American Type Culture Collection (ATCC, U.S.A.). The cells were cultured in DMEM (Dulbecco's modified Eagle's Medium) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were used at passage levels 30-45. For adhesion assays, monolayers of the Caco-2 cells were prepared on coverslips (Nunc™, U.S.A.), which were placed in 24-well Corning tissue culture plates. Cells were seeded at a concentration of $1-2\times10^5$ cells to obtain confluence. The culture medium was changed daily. Cultures at post-confluence after 7 days of culture were used. All experiments and maintenance of cells were carried out at 37°C in a 5% CO_2/95% air atmosphere.

Adhesion Assay of Bifidobacteria

Bifidobacterial strains were grown for 24 h at 37°C under anaerobic condition in the MRS medium containing 0.05% L-cysteine·HCl. The Caco-2 cell monolayers were washed twice with PBS (phosphate buffered saline, pH 7.4) buffer. For each adhesion assay, *Bifidobacterium* suspension ($1-3\times10^9$ cfu/ml) was mixed with an appropriate volume of cell-line culture medium and the mixture was added to each well of the cell culture plate which contained the coverslip, and it was then incubated for 1 h at 37°C in a 5% CO_2/95% air atmosphere. After incubation, the medium and nonadherent bacteria were removed by washing five times with PBS. The cell layer and remaining adherent bacteria were then fixed with methanol for 10 min. After fixation, the remaining cells were stained with Gram-stain, and examined microscopically under oil immersion. Each adhesion assay was conducted in duplicate with cells from three successive passages. For each coverslip monolayer, the number of adherent bacteria was counted in 10 random microscopic areas. Adhesion of bifidobacteria was expressed as the number of bacteria adhering to 50 Caco-2 cells.

CSH (Cell Surface Hydrophobicity) Assay

The CSH assay was performed according to Perez *et al.* [17]. Bacterial cells were grown to stationary phase, collected by centrifugation at 2,600 ×g for 15 min, washed twice in PBS, and resuspended in PBS to initial absorbance of OD=0.9±0.05. Three ml of bacterial suspensions were vortexed with 1 ml of xylene for 1 min and the phases were allowed to separate for 20 min. The CSH was calculated by using absorbance values of the aqueous phase before and after mixing with xylene, according to the equation:

$$CSH\ (\%)=100\times(A_i-A_f)/Ai$$

A_i, initial absorbance; A_f, final absorbance.

The higher CSH (%) means that more cells are partitioned from the aqueous phase into the xylene phase.

Enzymatic and Chemical Treatments of the Cultured Cells

To characterize the bacterial determinants involved in *Bifidobacterium* sp. BGN-4 adhesion, the cultured bacterial cells were subjected to various treatments. For EGTA treatment, the bacterial suspension was incubated with the Caco-2 cells in the presence of EGTA (20 mM). Wherever indicated, after monolayers were incubated with *Bifidobacterium* sp. BGN-4, the cells were washed five times with EGTA (20 mM) in PBS. For the heat treatment, bacterial cells were heated at 50, 55, and 60°C for 30 min in a water bath and cooled by immersion in an ice bath. For the protease treatment, bacterial cells were incubated with trypsin (2.5 mg/ml), pronase (2.5 mg/ml), and proteinase K (2.5 mg/ml) for 90 min at 37°C. After treatment, the enzymes were removed by washing 5 times with PBS. After the above various treatments, the treated cells were reacted with cultured Caco-2 cells and the adhesion assay was performed. The effect of sugar was determined by adding D-mannose, L-fucose, or D-galactose into the assay (100 mM, final concentration) medium. All the reagents were from Sigma-Aldrich (St. Louis, U.S.A.).

Scanning Electron Microscopy

Cells for scanning electron microscopy were grown on coverslips. After bacterial colonization assays, the cells were fixed with 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2-3 h at 4°C. After two washes with the same buffer, cells were post-fixed for 2 h with 1% (w/v) OsO_4 in the same buffer. The samples were dehydrated in graded series (30, 50, 70, 80, 95, 100%) of ethanol for 10 min each and passaged through HMDS (hexa methyl disilazame) twice for 15 min each. Cells were dried in hoods and coated with gold. The specimens were then examined with a scanning electron microscope (JEOL, JSM-5410LV, Japan).

Statistical Analysis

Data were analyzed by Duncan's multiple range test. A probability of $P<0.005$ in the two-tailed test was used in the criterion for statistical significance.

RESULTS AND DISCUSSION

Adhesion of *Bifidobacterium* to Enterocyte-Like Caco-2 Cells

The experimental human intestinal cell line Caco-2, a well-characterized cellular model established from a human colonic adenocarcinoma by Fogh *et al.* [7], spontaneously develops characteristics of mature enterocytes with functional brush-border microvilli with an apical membrane and a basolateral membrane that is separated by tight junctions [18]. Adhesion of bifidobacteria onto Caco-2 cells was compared with different strains *in vivo* and *in vitro* [5] and it was shown that the ability of the strain to adhere and colonize the intestinal cells *in vitro* and *in vivo* were similar. In the present study, the adhesion of the various *Bifidobacterium* strains to human enterocyte-like Caco-2 cells was compared. As listed in Table 1, there were considerable variations in their adherence to the differentiated Caco-2 cells among different strains, indicating that adhesive properties are not a universal feature of *Bifidobacterium*. Crociani *et al.* [5] also reported that bifidobacteria adhesion was very heterogeneous between strains of the same genus. Among the *Bifidobacterium* strains tested, *Bifidobacterium* sp. BGN-4 showed the greatest adherence to Caco-2 cells (Table 1). Scanning electron microscopy was used to visualize the physical interaction between *Bifidobacterium* sp. BGN-4 and the surface of cultured human intestinal Caco-2 cells (Fig. 1): The morphology of *Bifidobacterium* sp. BGN-4 showed a typical irregular rod and mostly aggregative forms. Adhesion of BGN-4 in the apical brush-border of the enterocytic Caco-2 cells, and only the bacteria surface facing the microvilli being involved in the adhesion. The BGN-4 interacted with the well-defined apical microvilli of Caco-2 cells without cell damage.

Table 1. Adhesion of the *Bifidobacterium* to Caco-2 cells.

Bifidobacterium strains	Adherent bacteria*
BGN-4	500
E2-18	168
E-15	132
JS-9	84
RD-54	67
SI	61
SH-2	47
RD-60	42
B. bifidum ATCC 2952	35
CN-2	30
SJ-32	26
KJ	24
HJ-30	23
B. animalis ATCC 2552	22
SH-5	17
B. adolescentis ATCC 15703	19
B. infantis ATCC 15697	15
B. longum ATCC 15707	11
M-6	10
MS-1	10

*Mean numbers of adhering bifidobacteria per 50 Caco-2 cells.

CSH of the *Bifidobacterium* Strains

In order to further characterize cell-cell interactions, a correlation between CSH and adhesion was examined, since it was suggested that the CSH and zeta potentials account for the attractive and repulsive forces, respectively, taking place in autoaggregation and adhesion of bacteria to different surfaces [9]. The CSH values of the *Bifidobacterium*

(A) (B)

Fig. 1. Adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells observed by scanning electron microscopy.
Notice that *Bifidobacterium* sp. BGN-4 whole cells interact with apical microvilli of Caco-2 cells [magnification ×15,000 (A) and ×20,000 (B)].

Table 2. Cell surface hydrophobicity of the *Bifidobacterium* strains.

Bifidobacterium strains	CSH (%)
BGN-4	93
KJ	90
HJ-30	90
B. adolescentis ATCC 15703	90
B. animalis ATCC 2552	86
M-6	85
RD-60	69.6
SI	66.3
CN-2	21
B. bifidum ATCC 2952	12
RD-54	7
MS-1	6
SH-5	6
E-15	5
E2-18	-
JS-9	-
SH-2	-
SJ-32	-
B. infantis ATCC 15697	-
B. longum ATCC 15707	-

-: Below the cell surface hydrophobicity value of 5%.

strains examined are presented in Table 2. The CSH of the different *Bifidobacterium* differed considerably among strains. Interestingly, *Bifidobacterium* sp. BGN-4, which showed the highest adherence to Caco-2 cells, also had the greatest CSH value. However, CSH of the overall strains was not closely interrelated with the Caco-2 adhesion. Wadstrom *et al.* [23] reported that some strains in lactobacilli, despite their hydrophilic surface properties, were capable of adhering, suggesting that multiple mechanisms are involved in the adhesion process. Perez *et al.* [17] reported that strains with a nearly identical hydrophobicity value had a wide range of adherence levels. This implies that other factors may also modulate the adhesion that is driven by hydrophobicity.

The Effects of Growth Temperature of the *Bifidobacterium* sp. BGN-4 on CSH and Adherence to Caco-2 Cells

To examine the effect of growth temperature on CSH and adherence to Caco-2 cells, *Bifidobacterium* sp. BGN-4 was used. The CSH and adhesion to Caco-2 cells of *Bifidobacterium* sp. BGN-4 decreased gradually when the growth temperature increased from 30 to 42°C (Fig. 2). When BGN-4's growth temperature was 42°C, *Bifidobacterium* sp. BGN-4's morphology of adhesion to Caco-2 cells changed from aggregative to diffuse (data not shown). The results suggested that CSH of *Bifidobacterium* sp. BGN-4 may be somewhat related to the Caco-2 adhesion. The significance of the CSH of the *Bifidobacterium* to their ability to survive and adapt in the environment needs to be studied further.

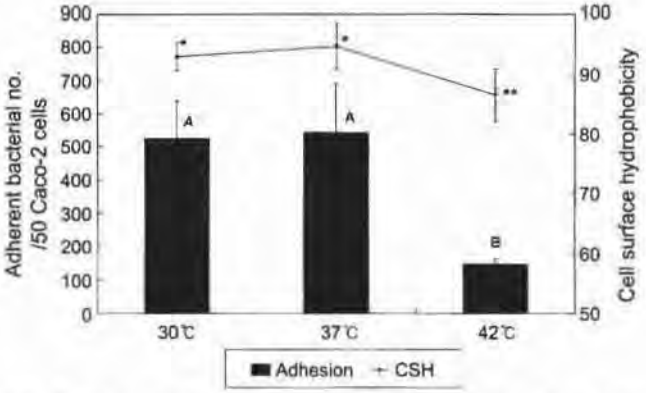

Fig. 2. The effects of growth temperature on cell surface hydrophobicity and adherence to Caco-2 cells of the *Bifidobacterium* sp. BGN-4.

Data are means±SD of triplicate cultures. Values with different superscripts or alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

The Effects of Heat Treatment of *Bifidobacterium* sp. BGN-4 Cells on the Adhesion to Caco-2 Cells

In order to examine the effect of heat temperature on the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells, the adhesion of the heat-treated bifidobacterial cells at different temperatures was compared. As shown in Fig. 3, the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells gradually decreased as heat-treatment temperature increased. Heat treatment at 60°C almost completely abolished the adhesiveness of *Bifidobacterium* sp. BGN-4. This result suggests that the factor(s) involved in the adhesion of *Bifidobacterium* sp. BGN-4 was found to be heat-sensitive.

The Effects of Various Enzymatic and Chemical Treatments on the Adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 Cells

The presence of simple sugars such as fucose, galactose, or mannose in the adhesion assay mixture decreased the

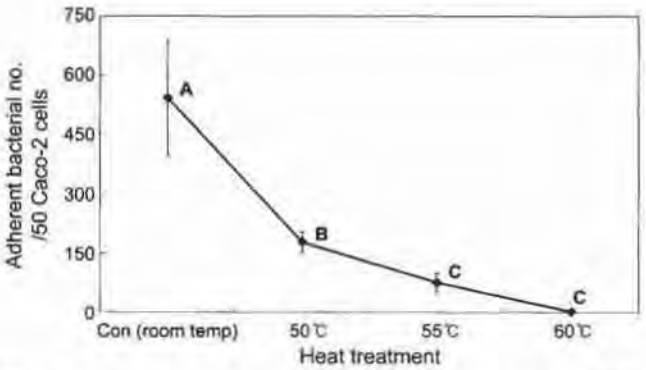

Fig. 3. The effects of heat treatment of *Bifidobacterium* sp. BGN-4 cells on the adhesion to Caco-2 cells.

Data are means±SD of triplicate cultures. Values with different alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

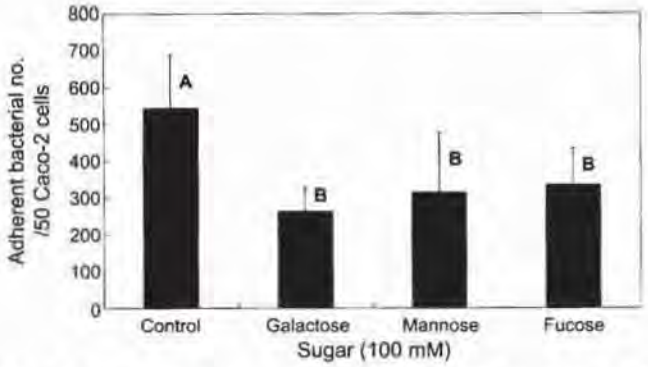

Fig. 4. The effects of simple sugars on the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells.
Data are means±SD of triplicate cultures. Values with different alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

adhesion of *Bifidobacterium* sp. BGN-4 by as much as 50%, however, there was no difference between the sugars (Fig. 4). Greene and Klaenhammer [11] showed that enhanced adhesion of lactobacilli to intestinal cells was promoted by the divalent cation calcium, possibly due to an ionic bridge between the surfaces of bacteria and epithelial cells. In this study, treatment of *Bifidobacterium* sp. BGN-4 with EGTA, which is a calcium-chelating agent, did not induce any significant change in the degree of adhesion of *Bifidobacterium* BGN-4 to Caco-2 cells (data not shown). The calcium dependency may vary among different strains, since Chauvier *et al.* [4] reported that, among the adhering lactobacilli of ten strains, five strains had high calcium independent binding capacity to Caco-2 cells. The contribution of protein factors attached to the cell walls was examined by treating the bacterial cells with three proteolytic enzymes; Trypsin, pronase, and proteinase K treatments of bacterial cells with these enzymes had different degree of effects, but always reduced the adhesion of *Bifidobacterium* sp. BGN-4 (Fig. 5). This strongly suggests that the adhesion of *Bifidobacterium* sp. BGN-4

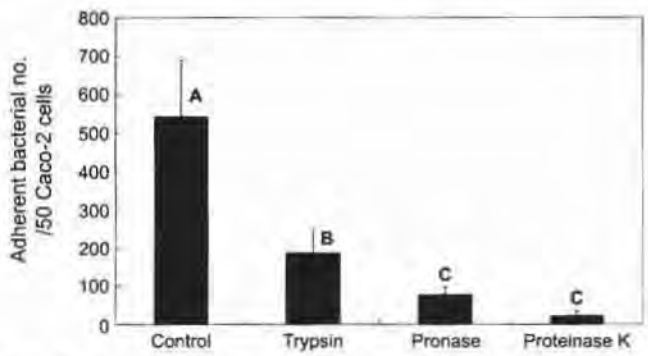

Fig. 5. The effects of proteolytic enzymes on the adhesion of *Bifidobacterium* sp. BGN-4 to Caco-2 cells.
Data are means±SD of triplicate cultures. Values with different alphabets were significantly different at $P<0.05$ by Duncan's multiple range test.

is mediated to some extent by a proteinaceous component of bacterial origin. Also, the involvement of nonproteinaceous cell wall component, lipoteichoic acids (LTA) of Gram-positive bacteria, in the binding to epithelial cells was reported. For example, Op den Camp *et al.* [14] reported that binding of the lipoteichoic acids of *Bifidobacterium bifidum* to human colonic epithelial cells appears to be specific, reversible, and cell concentration and time dependent, and suggested that ester-linked fatty acids are essential for LTA-binding. Thus, the differences in the capacity of adhesion observed in our present study might reflect the strain differences in the physiology and content of the different adhesion factors; i.e, proteinaceous component, polysaccharide, ionic charge, or lipoteichoic acid. BGN-4 showing the strongest adhesion to the epithelial cell among the strains tested, and so may be a good candidate for a probiotic strain in order to improve the human intestinal microbial balance.

Acknowledgments

This work was supported by grants received from Korean Ministry of Science and Technology (2000-BT-28) and Korean Ministry of Commerce, Industry and Energy (A11-29-03).

References

1. Bernet, M. F., D. Brassart, J. R. Neeser, and A. L. Servin. 1993. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. *Appl. Environ. Microbiol.* **59:** 4121-4128.
2. Biavati, B., B. Sgorbati, and V. Scardovi. 1992. The genus *Bifidobacterium*, pp. 816-833. *In* Balows, A., Trupen, H. G., Dworkin, M., Harder, W. and Schlifer, K.-H. (eds.). *The Prokaryotes. A Handbook on Habitats, Isolation, and Identification of Bacteria*. Springer-Verlag, New York, U.S.A.
3. Blomberg, L., A. Henriksson, and P. L. Conway. 1993. Inhibition of adhesion of *Escherichia coli* K 88 to piglet ileal mucus by *Lactobacillus* spp. *Appl. Environ. Microbiol.* **59:** 34-39.
4. Chauviere, G., M. H. Coconnier, S. Kerneis, J. Fourniat, and A. L. Srevin. 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. *J. Gen. Microbiol.* **138:** 1689-1696.
5. Crociani, J., J. P. Grill, M. Huppert, and J. Ballongue. 1995. Adhesion of different bifidobacteria strains to human enterocyte-like Caco-2 cells and comparison with *in vivo* study. *Lett. Appl. Microbiol.* **21:** 146-148.
6. Del Re, B., A. Busetto, G. Vignola, B. Sgorbati, and D. L. Palenzona. 1998. Autoaggregation and adhesion ability in a

Bifidobacterium suis strain. *Lett. Appl. Microbiol.* **27:** 307-310.
7. Fogh, J., J. M. Fogh, and T. Orfeo. 1977. One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Res.* **59:** 221-226.
8. Fontaine, I. F., E. A. Aissi, and S. J. L. Bouquelet. 1994. *In vitro* binding of *Bifidobacterium bifidum* DSM 20082 to mucosal glycoproteins and hemagglutinating activity. *Curr. Microbiol.* **28:** 325-330.
9. Geertsema-Doornbusch, G. I., H. C. van der Mei, and H. J. Busscher. 1993. Microbial cell surface hydrophobicity. The involvement of electrostatic interactions in microbial adhesion to hydrocarbons (MATH). *J. Microbiol. Methods* **18:** 61-68.
10. Gibson, G. R. and X. Wang. 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *J. Appl. Bacteriol.* **77:** 412-420.
11. Greene, J. D. and T. R. Klaenhammer. 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. *Appl. Environ. Microbiol.* **60:** 4487-4494.
12. Lee, M. J., Z. Zang, E. Y. Choi, H. K. Shin, and G. E. Ji. 2002. Cytoskeleton reorganization and cytokine production of macrophages by bifidobacterial cells and cell-free extracts. *J. Microbiol. Biotechnol.* **12:** 398-405.
13. Mitsuoka, T. 1984. *A Color Atlas of Anaerobic Bacteria*, 2nd Ed. pp. 319-327.
14. Op den Camp, H. J., A. Oosterhof, and J. H. Veerkamp. 1985. Interaction of bifidobacterial lipoteichoic acid with human intestinal epithelial cells. *Infect. Immun.* **47:** 332-334.
15. Park, M. S., K. H. Lee, and G. E. Ji. 2001. Molecular cloning and characterization of the β-galactosidase gene from *Bifidobacterium adolescentis* Int57. *J. Microbiol. Biotechnol.* **11:** 106-111.
16. Park, S. Y., G. E. Ji, Y. T. Ko, H. K. Jung, U. Zeynep, and J. J. Pestka. 1999. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. *Int. J. Food Microbiol.* **46:** 231-241.
17. Perez, P. F., Y. Minnaard, E. A. Disalvo, and G. L. D. Antoni. 1998. Surface properties of bifidobacterial strains of human origin. *Appl. Environ. Microbiol.* **64:** 21-26.
18. Pinto, M., S. Robine-Leon, M. D. Appray, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47:** 323-320.
19. Rasic, J. L. and J. A. Kurmann. 1983. *Bifidobacteria and their Role*. Birkhauser Verlag, Basel, Switzerland.
20. Sekine, K., J. Ohta, M. Onishi, T. Tatsuki, Y. Shimokawa, T. Toida, T. Kawashima, and Y. Hashimoto. 1995. Analysis of antitumor properties of effector cells stimulated with a cell wall preparation (WPG) of *Bifidobacterium infantis*. *Biol. Pharm. Bull.* **18:** 148-153.
21. Sgorbati, B., B. Biavati, and D. Palenzona. 1995. The genus *Bifidobacterium*, pp. 279-306. *In* Wood, B. J. B. and W. H. Holzapfel (eds.). *The Lactic Acid Bacteria*, Vol. II. Chapman and Hall Academic and Professional, Sweden.
22. Tissier, H. 1900. Recherches sur la flore intestinale normale et pathologique du nourisson. Thesis. University of Paris, France.
23. Wadstrom, T., K. Andersson, M. Sydow, L. Axelsson, S. Lindgren, and B. Gullmar. 1987. Surface properties of lactobacilli isolated from the small intestine of pigs. *J. Appl. Bacteriol.* **62:** 513-520.

Food Sci. Biotechnol. Vol. 12, No. 2, pp. 178~179 (2003)

RESEARCH NOTE

Food Science and Biotechnology

Effect of *Bifidobacterium*-fermented Soy Hypocotyls Intake on the Composition of Human Large Intestinal Bacteria in the Elderly

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Abstract Effect of dietary supplementation of *Bifidobacterium*-fermented soy hypocotyls (BFSH) on the composition of large intestinal bacteria of the elderly people was investigated. Four grams of BFSH containing 10^9 cfu/g *Bifidobacterium* were administered daily to 14 elderly volunteers every day for 10 days, followed by 10 days of non-intake period, and the cycle was repeated for 50 days. Composition of the intestinal bacteria (*Bacteroides, Bifidobacterium, Lactobacillus, E. coli, Clostridium perfringens*) examined revealed that administration of BFSH resulted in a marked increase in *Bifidobacterium* and a decrease in *Bacteroides*. Stool evacuation frequencies, pH, and water contents of the fecal samples did not change significantly.

Keywords: *Bifidobacterium*, soy, intestinal bacteria, elderly

Introduction

The human intestinal tract contains a complex and dynamic bacterial population, whose cell numbers amount to 10^{14} cfu (colony forming units) per gram (1). The metabolic activities of the bacteria can exert both harmful or beneficial effects on the human host depending on the species, diet, and other various environmental factors (1). The number of putrefactive bacteria such as *Clostridium perfringens* are known to increase with the aging of the human host, leading to increased production of ammonia, H_2S, and amines, which can aggravate the intestinal environment of the elderly people (2). On the other hand, the number of beneficial bacteria such as *Bifidobacterium* decreases at a significant level during aging. Upon examining the composition of the intestinal bacteria of the Koreans, Ji reported that number of *Bifidobacterium* was lower and that of *Cl. perfringens* higher in the elderly people (above 65 years group) than the young people (3). In spite of the recent marked increase in elderly people in Korea, little studies have been conducted to improve the health state in terms of the intestinal bacterial balance for the elderly people. Probiotics are well known to affect the composition of indigenous microflora and may have several beneficial effects on the human health such as the maintenance of a balanced flora, alleviation of lactose intolerance symptom, and resistance to enteric pathogens (4). Modes of action of probiotics are suggested to be the colonization of the gastro-intestinal tract, prevention of pathogen overgrowth, neutralization of enterotoxins, modulation of the activity of bacterial enzymes in the large intestine, improvement of the digestive capacity of the small intestine, and adjuvant effect on the immune system (5). In most cases, clinical studies were preceded by laboratory-based research, utilizing animal models or *in vitro* culture systems, to provide preliminary evidence on the intestinal condition. In this study, soybean hypocotyls, part of the axis of soybean embryo below the cotyledons, were fermented with *Bifidobacterium*. The effect of dietary supplementation of *Bifidobacterium*-fermented soy hypocotyls (BFSH) on the intestinal bacteria of the elderly people was investigated.

Materials and Methods

Soybean hypocotyls were ground and suspended in 10 volumes of water. The soy hypocotyls medium was flushed with gas mixture (95% N_2 and 5% CO_2) to remove dissolved oxygen. For fermentation, *Bifidobacterium* sp. BGN4 previously characterized (6) was used. After autoclaving, fresh *Bifidobacterium* sp. BGN4 cultures were inoculated at a 1:50 ratio. The suspension fermented for 24 hr was lyophilized until dryness. It contained *Bifidobacterium* counts of 10^9cfu/g dry weight. Four grams (two grams after breakfast and two grams after dinner) of the powder were administered to 14 elderly volunteers (7 each males and females) every day for 10 days, followed by 10 days of non-intake period. The nonintake-intake cycle was repeated for 50 days. During the study period, all subjects were asked to avoid consumption of fermented food and other probiotic products. The composition of intestinal bacteria was examined at the last day of each period using fresh fecal samples. The serially diluted samples were plated on various selective and non-selective media as described by Ji (3). For counting anaerobic organism, Anoxomat (MART, Netherland) was used to equilibrate

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Table 1. Effect of BFSH intake on the composition of human feces

	Non-intake / Intake cycle				
	Before intake	First intake	First non-intake	Second intake	Second non-intake
Microorganism	(Log of CFU per gram wet feces)				
Bifidobacterium	9.0±0.5	9.5±0.5	9.0±0.8	10.0±0.4	9.1±0.8
Bacteroides	9.6±0.4	8.7±1.5	10.1±0.4	9.7±0.3	9.9±0.6
Lactobacillus	ND	7.2±1.4	7.5±1.3	8.0±1.4	7.5±1.2
Clostridium	4.5±0.7	4.8±1.3	5.1±0.6	4.7±0.9	5.3±1.1
E. coli	8.6±0.5	7.6±1.0	7.8±1.0	7.9±2.3	7.6±0.8
Total aerobic bacteria	8.8±0.4	7.5±1.0	8.1±0.8	8.4±0.6	8.4±0.5
Water content (%)	73.6±8.6	70.2±9.1	70.2±8.5	71.7±9.2	73.2±8.9
pH	6.9±0.6	6.8±0.8	6.8±0.9	6.9±1.0	6.5±0.6

All numbers in Mean±S.D. ND means not determined.

the anaerobic chamber and the plate was incubated 2-3 days at 37°C. The pH was measured by a pH meter (Fisher Model 10, USA) after the suspension of fecal samples with 4 volumes of water. The moisture contents were analyzed after drying at 105°C in a drying oven. Group means comparisons were tested for significance by Student's *t*-test. Statistical significance was defined as $P<0.05$. Fecal composition results of the subjects are expressed as Means±S.D..

Results and Discussion

Bioconversion of soy oligosaccharides and isoflavones, and their corresponding enzymes, alpha-galactosidase and beta-glucosidase, has been previously characterized in our laboratory during soy fermentation using *Bifidobacterium* (7, 8). For the present study, we used a hypocotyl portion of the soy for fermentation. The effect of dietary supplementation with BFSH on intestinal bacteria of the elderly people is shown in Table1.

Among the examined bacteria, viable *Bifidobacterium* cell number showed the most pronounced increase during intake, whereas that of *Bacteroides* decreased following the consumption of BFSH. No significant changes were obtained for *E, coli, Lactobacillus,* and *Cl. perfringens*. *Bifidobacterium* comprising more than 90% of the intestinal bacteria in breast-fed infants decreases down to about 10% after weaning and during the following life time period (1). *Bifidobacterium* has been considered to confer various beneficial effects on the human hosts, whereas *Bacteroides* are known to produce various putrefactive compounds and are the most frequently found anaerobic infectious agents in various tissues aside from the intestine (1, 2). In this context, our result may be interpreted as an improvement in the balance of the intestinal bacteria by the supplementation of BFSH. Further analysis needs to be performed on whether the observed effects were solely due to administered *Bifidobacterium* or whether some other components of BFSH are responsible. The effect of BFSH intake on pH, moisture contents, and stool evacuation frequencies were not eminent. Throughout the experiment, the participants showed a high interest in the experiments and willingness to donate their fecal samples. More research on the development of the probiotic foods for the elderly is warranted considering that the population of the elderly will soon double by year 2020. Further studies should delineate the mechanism of the probiotic action, the evaluation of the physiological effect, and the efficient delivery method of probiotic products for special targets (e.g., age group, intestinal organ, etc.) in more detail.

Acknowledgments

This work was supported by a grant from the Korean Ministry of Science and Technology (2000-BT-78).

References

1. Cummings, J.H. and Macfarlane, G.T. The control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. 70: 443-459 (1991)
2. Mitsuoka, T. Recent trends in research on intestinal flora. Bifidobact. Microfl. 1: 3-24 (1982)
3. Ji, G.E. Composition and distribution of intestinal microbial flora in Korean. Korean J. Appl. Microbiol. Biotechnol. 22: 453-458 (1994)
4. Vanderhoof J.A. Probiotics: future directions. Am. J. Clin. Nutr. 73: 1152S-1155S (2001)
5. Raibaud, P., Raynand, J.P., Metz, J.H.M. and Groenestein, C.M. Experimental data on the mode of action of probiotics. In: Proceedings Int. Symp. on Veal Calf Production, Wagenigen, The Netherlands. 14-16 March. pp. 269-275 (1990)
6. Park, S.Y., Ji, G.E., Ko, Y.T., Jung, H.K., Zeynep, U. and Pestka, J.J. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. Int. J. Food Microbiol. 46: 231-241. (1999)
7. Kwon, B., Kim, Y.B., Lee, J.H., Lee, H.J., Chung, D.K. and Ji, G.E. Analysis of sugars and alpha-galactosidase activity during soymilk fermentation by bifidobacteria. Food Sci. Biotechnol. 11: 389-391 (2002)
8. Jeon, K.S., Ji, G.E. and Hwang, I.K. Assay of beta-glucosidase activity of bifidobacteria and the hydrolysis of isoflavone glycosides by *Bifidobacterium* sp. Int-57 in soymilk fermentation. J. Microbiol. Biotechnol. 12: 8-13 (2002)

(Received October 21, 2002; accepted February 17, 2003)

FEMS Microbiology Letters 240 (2004) 131–136

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from *Bifidobacterium bifidum* BGN4

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Received 17 May 2004; received in revised form 17 September 2004; accepted 17 September 2004

First published online 28 September 2004

Edited by W. Kneifel

Abstract

Strains of bifidobacteria have many health-promotion effects. Whole cells or cytoplasm extracts of *Bifidobacterium bifidum* BGN4, isolated from human feces, inhibited the growth of several cancer cell lines. The polysaccharide fraction (BB-pol) extracted from *B. bifidum* BGN4 had a novel composition, comprising chiroinositol, rhamnose, glucose, galactose, and ribose. Three human colon cancer cell lines were treated with BB-pol: HT-29, HCT-116, and Caco-2. Trypan blue exclusion assay and BrdU incorporation assay showed that BB-pol inhibited the growth of HT-29 and HCT-116 cells but did not inhibit the growth of Caco-2 cells.

Keywords: *Bifidobacterium*; Chiroinositol-containing polysaccharide; Colon cancer; Growth inhibition; Probiotics

1. Introduction

Probiotics are traditionally defined as live microbial food supplements which beneficially affect the host animal by improving its intestinal microbial balance [1]. In view of their perceived health-promotion effects [2,3], probiotic bacteria have been increasingly included in various types of food products (especially fermented milks) during the past two decades [4].

There is experimental evidence that probiotic microorganisms show an anticancer activity in vitro and in animal models. Pool-Zobel et al. reported that *Lactobacillus acidophilus*, *L. gasseri*, *L. confusus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, and *B. longum* were antigenotoxic toward *N*′-nitro-*N*-nitrosoguanidine- or 1,2-dimethylhydrazine-induced genotoxicities [5]. Other studies have shown that certain strains of lactic acid bacteria (LAB) prevent putative preneoplastic lesions or tumors induced by carcinogens such as 1,2-dimethylhydrazine or azoxymethane [6–9]. Many strains – such as *L. rhamnosus* GG [10], *L. acidophilus* [11], *L. casei*, *B. longum* [12,13], *B. infantis*, *B. adolescentis*, and *B. breve* – showed significant suppression of colon tumor incidence in this type of study. In addition, there is direct evidence for antitumor activities of LAB obtained in studies using preimplanted tumor cells in animal models. There are several reports [14,15] that the consumption of fermented milk and/or cultures containing LAB or the intralesional injection of live or dead *Bifidobacterium* cells inhibited the growth of tumor cells injected into mice.

The precise mechanisms by which LAB inhibit colon cancer are presently unknown. However, based on

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0378-1097/$22.00
doi:10.1016/j.femsle.2004.09.020

experimental and epidemiological studies [3,16,17], several mechanisms have been proposed, including (1) enhancing the host's immune response, (2) binding and degrading potential carcinogens, (3) qualitative alterations in the intestinal microflora incriminated in producing putative carcinogens and promoters (e.g., bile-acid-degrading bacteria), (4) production of antitumorigenic or antimutagenic compounds in the colon, and (5) alteration of metabolic activities of intestinal microflora.

Various types of LAB preparations showed antitumor activities. Sekine et al. [18] found antitumor activity in peptidoglycans isolated from *B. infantis* strain ATCC 15697, and Oda et al. [19] reported antitumor polysaccharide fractions originating from *Lactobacillus* cultures. Glycoproteins found in the supernatants of *Lactobacillus* cultures have also shown antitumor effects [20].

The present study analyzed the composition of polysaccharide (BB-pol) extracted from *B. bifidum* BGN4 and investigated the effects of BB-pol on human colon cancer cell lines. We examined the potential of BB-pol as an antiproliferation compound using a direct cell counting method and measuring the rate of DNA synthesis.

2. Materials and methods

2.1. Bacterial culture and preparation of cytoplasm extract

B. bifidum BGN4 and other bifidobacteria which were isolated from the feces of healthy human subjects and previously reported by Park et al. [21] were used for the experiments. *B. bifidum* BGN4 was cultured and subcultured anaerobically in MRS broth (Criterion, USA) containing 0.5% L-cysteine (Sigma, USA) at 37 °C for 20 h. For the preparation of bacterial cytoplasm extracts, cultured bacterial cells were collected by centrifugation (Hanil, Korea) at 1390*g* for 20 min at 4 °C, and washed twice with autoclaved phosphate-buffered saline, followed by final washing with autoclaved double-distilled water. Washed whole cells were disintegrated using a cell disruptor, and the cell pellets were removed by centrifugation at 1390*g* for 20 min at 4 °C. The supernatant was centrifuged at 22,250*g* for 45 min at 4 °C for the separation of cell walls and cytoplasm extract. The sediment (cell-wall fraction) was removed, and the supernatant was used as the cytoplasm extract. This fraction was lyophilized (Ilshin, Korea) and stored at −70 °C until used.

2.2. Isolation of polysaccharide from bifidobacterial cytoplasm extract

The lyophilized cytoplasm extract was dissolved in autoclaved double-distilled water and heat-treated at 80 °C for 30 min in a water bath (Jeio Tech, Korea). Protein denaturing due to the heat treatment made the solution hazy. The sample was centrifuged until the supernatant turned clear, then phenol solution was added to the collected supernatant at a ratio of 1:1 (v/v) for protein denaturalization. After the end of the reaction with phenol, the solution was mixed vigorously and centrifuged again. The collected supernatant was treated with a phenol–chloroform solution at a ratio of 1:1 (v/v). Treatment of the collected supernatant with the phenol–chloroform solution was repeated. Lastly, chloroform solution was added at a ratio of 1:1 (v/v), and the collected supernatant was precipitated with cold ethanol, followed by freeze-drying. A phenol–sulfuric acid assay was used to determine whether this sample was the polysaccharide fraction.

2.3. Analysis of BB-pol composition

For analyzing the composition of the polysaccharide fraction, it was hydrolyzed by trifluoroacetic acid. The polysaccharide was reacted with trifluoroacetic acid at room temperature for 30 min and then reacted at 100 °C for 150 min. The composition of the hydrolyzed polysaccharide was determined by a Bio-LC with an electrochemical detector (Dionex ED-50, USA) using a Dionex CarboPac MA1 column with 600 mM sodium hydroxide at a flow rate of 0.4 $ml\,min^{-1}$. The composition of the polysaccharide was confirmed with standard samples of chiroinositol, rhamnose, glucose, galactose, and ribose (Sigma, USA).

2.4. Cell culture

This study used three human colon cancer cell lines (Table 1): HT-29 and HCT-116 cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea), and the Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, USA). They were grown in DMEM culture media supplemented with 10% (v/v)

Table 1
The characteristics of cell lines used in this study (KCLB, Korean Cell Line Bank; ATCC, American Type Culture Collection)

Cell line	Tissue	Species	Growth property	KCLB (ATCC) no.
HT-29	Colon or colorectal, adenocarcinoma	Human	Adherent	KCLB 30038
HCT-116	Colon or colorectal, carcinoma	Human	Adherent	KCLB 10247
Caco-2	Colon or colorectal carcinoma	Human	Adherent	HTB-37

fetal bovine serum (Gibco BRL, USA) and 1% antibiotic–antimycotic solution (Gibco BRL, USA). All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. After they were grown to confluence in sterile tissue culture plates (100 mm × 20 mm; Corning, USA), cells were detached and transferred to the new cell culture dishes using trypsin EDTA (Gibco BRL, USA) for each experiment. The detached cells were seeded to a 96-well cell culture cluster (at a density of 5×10^3 cells per well), 12 wells ($30–50 \times 10^3$ cells per well), or culture plates (60 mm × 15 mm; $100–200 \times 10^3$ cells per plate). They were then incubated for 48 h in the presence of 0, 20, 40, or 80 $\mu g\,ml^{-1}$ BB-pol.

2.5. Trypan blue staining

Cell numbers and their viability were assessed by the trypan blue dye-exclusion method [22]. Cancer cells were seeded onto 12 multiwell plates at a density of $30–50 \times 10^3$ cells per well in serum-containing medium. All cells were allowed to attach for 12–24 h, and each well was treated with BB-pol at a concentration of 20, 40, or 80 $\mu g\,ml^{-1}$. At two days after BB-pol treatment, cultured cells were trypsinized and mixed with an equal volume of trypan blue solution (Sigma–Aldrich, UK). The mixture was loaded on a hemacytometer (Superior, Neubauer, Germany) and the stained/unstained cells were observed using an inverted microscope (BX-40, Olympus, Japan) at 100× magnification.

2.6. BrdU (5-bromo-2′-deoxyuridine) incorporation assay for measurement of DNA synthesis rate

As a microplate format, cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates, and also incubated for 48 h in the presence of 0, 20, 40, or 80 $\mu g\,ml^{-1}$ BB-pol.

In this study, a nonradioactive alternative to the [^{3}H]-thymidine incorporation assay was performed using a commercial cell proliferation ELISA kit (BrdU, colorimetric; Roche, Germany) according to the manufacturer's instructions. Briefly, at the time for assay (two days after BB-pol treatment), 10 μl of BrdU labeling solution was added to control and BB-pol-treated cells, and the plates were reincubated for 3 h for labeling. The cells were then denatured and fixed using Fix/Denat solution for 30 min at 37 °C, and anti-BrdU-POD was added to the plates and incubated for 90 min at 15–25 °C. Lastly, 100 μl of substrate solution was added to each well and the plates were incubated at 15–25 °C until color development was sufficient for photometric detection. Twenty-five microliters of 1 M H_2SO_4 was added as a stopping solution to each well, and absorbance was measured with an ELISA reader (Bio-Rad Laboratories, USA) at 450 nm.

2.7. Statistical analysis

Data were analyzed by a one-way ANOVA procedure of SAS software (SAS Institute Inc.,1999). The differences between mean values were detected by the Duncan's multiple range test.

3. Results and discussion

3.1. Selection of the best Bifidobacterium strain for inhibiting the growth of cancer cell lines

In order to determine if *Bifidobacterium* cells have an inhibitory effect on the growth of colon cancer cell lines, HT-29 cells were treated with heat-treated cells of 30 different bifidobacterial strains and trypan blue assay was performed. In this preliminary assay, *B. bifidum* BGN4 showed the greatest effect (data not shown). To further characterize the functional substances of *B. bifidum* BGN4, HT-29 cells were treated with different types of cell fraction. Among the whole-cell, cell-wall, and cytoplasm extracts, the cytoplasm extract showed the highest inhibitory effect.

3.2. Composition of BB-pol

The composition of the polysaccharide was determined by a Bio-LC using a Dionex CarboPac MA1 or PA1 column. The detected peaks were compared with various carbohydrate standards: chiroinositol, myoinositol, inositol, rhamnose, glucose, galactose, ribose, fructose, adonitol (ribitol), glycerol, mannose, arabinose, fucose, xylose, sorbose, tagatose, mannitol, sorbitol, dulcitol, xylitol, allose, psicose, glucuronic acid, glucaric acid, galacturonic acid, galactosamine, mannosamine, and glucosamine. The polysaccharide was found to consist of 26.4% chiroinositol, 3.9% rhamnose, 31.5% glucose, 11.0% galactose, and 23.8% ribose (Fig. 1). We think that the BB-pol fraction consisted of one polysaccharide because of a peak profilefor the BB-pol fraction in GPC. The molecular weight ofthe BB-pol fraction was about 1,500,000 by extrapolation using dextran standards (M_W, 48,500, 273,000, 830,000). It is interesting that BB-pol contained chiroinositol as one of the major sugar units, because a chiroinositol-based polysaccharide from microbial sources has not previously been reported.

3.3. Effect of BB-pol on the growth inhibition of HT-29 and HCT-116 cells

This study examined the effects of BB-pol on the growth of three types of human colorectal adenocarcinoma cell lines using a trypan blue exclusion assay (Fig. 2). BB-pol tended to decrease the numbers of

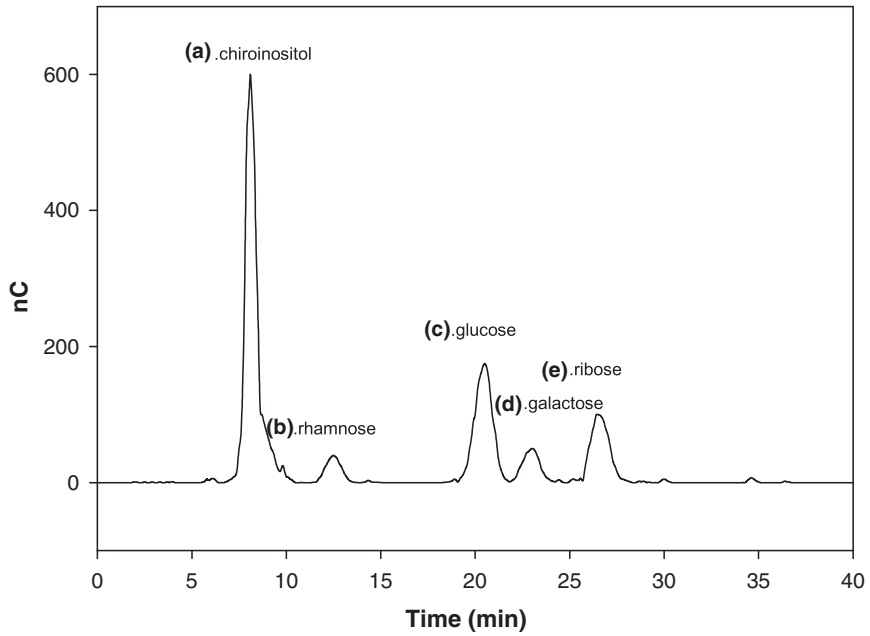

Fig. 1. Bio-LC analysis of the composition of BB-pol. (a) chiroinositol (26.4%); (b) rhamnose (3.9%); (c) glucose (31.5%); (d) galactose (11.0%); (e) ribose (23.8%).

HT-29 cells even at a concentration of 10 $\mu g ml^{-1}$. However, Caco-2 cells were not noticeably inhibited at concentrations up to 100 $\mu g ml^{-1}$ (data not shown). Although all of the three cell lines originated from human adenocarcinomas, Caco-2 is known to resemble the physiology of normal cells [23–25] more than the other two cell lines used in this study. This suggests that BB-pol had a greater effect on tumor cell lines with higher malignancy. Fig. 2 shows the growth inhibition caused by BB-pol in HT-29 and HCT-116 cells. BB-pol at 20 $\mu g ml^{-1}$ inhibited the growth of HT-29 cells by 50.5 ± 3.6% (mean ± SD). The growth inhibition of HCT-116 cells increased with the BB-pol concentration, but these cells were slightly less sensitive to BB-pol than HT-29 cells. Few, if any, cells became nonviable during 48 h of incubation, which suggests that the inhibition of tumor cell growth by BB-pol was due to the retardation of cell growth rather than cytotoxicity. This was further supported by an lactate dehydrogenase (LDH) release assay in which no significant difference was evident between the experimental groups (data not shown).

3.4. Effect of BB-pol on the growth inhibition of HT-29 and HCT-116 cells measured by BrdU incorporation assay

The trypan blue exclusion assay is a direct method for measuring cell growth or cytotoxicity, but it involves many steps that may introduce experimental errors. Therefore, a BrdU incorporation assay was performed to confirm the trypan blue staining data. The rationale of the BrdU incorporation assay was to measure the changed DNA synthesis of treated cells as for [^{3}H]-thymidine incorporation assay.

Data from BrdU incorporation assays in HT-29 and HCT-116 cells showed results similar to those of growth inhibition from the trypan blue exclusion assay (Fig. 3). Similar to the result of trypan blue staining, BB-pol had no effect on the DNA synthesis rate measured by the BrdU incorporation assay in Caco-2 cells (data not shown). In HT-29 cell lines, the DNA synthesis rate decreased after BB-pol treatment in a dose-dependent manner. When treated with 80 $\mu g ml^{-1}$ BB-pol, the adjusted DNA synthesis rate was 52.6 ± 0.9%. Treatment with the same concentration of BB-pol produced a smaller decrease in the DNA synthesis of HCT-116 cells: the adjusted DNA synthesis rate was 64.3 ± 1.9%. However, HT-29 and HCT-116 cells treated with 20 $\mu g ml^{-1}$ BB-pol showed similar reductions in DNA synthesis rates.

As shown in the former two assays, the decrease in the number of counted cells in the BB-pol-treated group was concordant with the reduced DNA synthesis rate. These experimental data allude to a growth-retarding effect of BB-pol on HT-29 and HCT-116 cell lines rather than cytotoxic effects.

In contrast, most previously reported polysaccharides that exhibit antitumor activities did not directly inhibit the growth of tumor cells in vitro, but instead exerted antitumor activity by stimulating macrophages and various immune systems. Such polysaccharides included α- and β-glucan from various mushrooms [26], SPR-901 α-glucan from rice bran [27], α-glucan from *Mycobacterium bovis*, Bacille Calmette–Gurin [28], and water-soluble polysaccharide containing glucose and galactose as

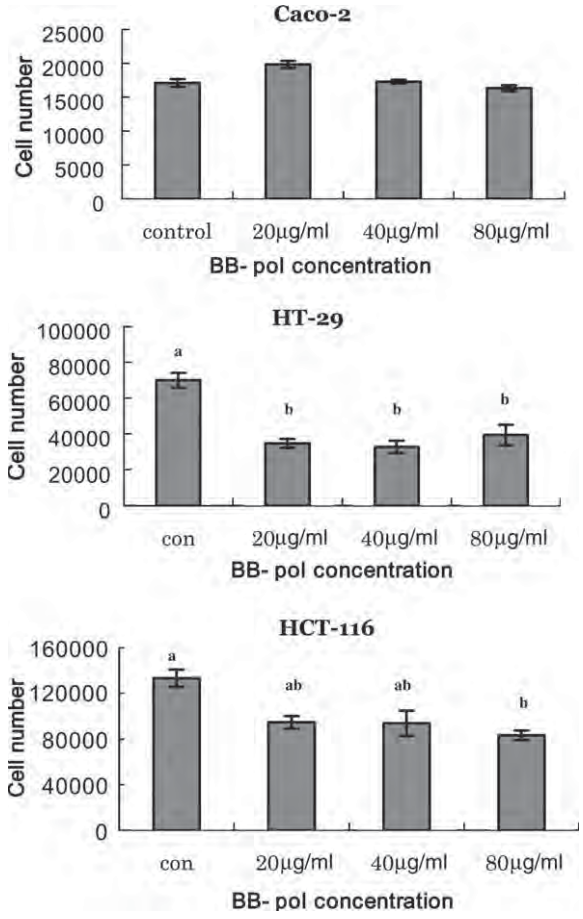

Fig. 2. Effect of BB-pol on the growth of human colon cancer cell lines. The cell lines were cultured for 48 h in the presence of BB-pol at various concentrations: 0 (control), 20, 40, and 80 $\mu g ml^{-1}$ in 12-well plates. The cell numbers were determined by trypan blue exclusion assay. Data values are mean ± SE of triplicates in a representative assay ($P < 0.05$).

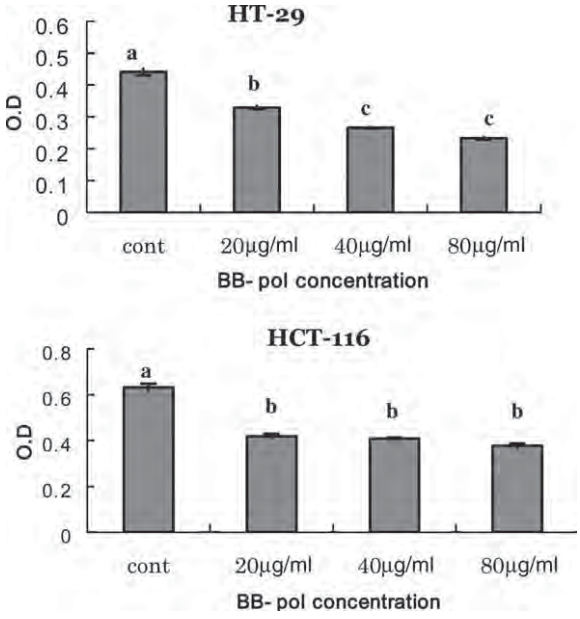

Fig. 3. Effect of BB-pol on the growth inhibition of HT-29 and HCT-116 cell lines measured by BrdU incorporation assay. The cells were cultured for 48 h in the presence of BB-pol at various concentrations in 96-well plates: 0 (control), 20, 40, and 80 $\mu g ml^{-1}$. The DNA synthesis rates were determined by BrdU incorporation assay. Data values are mean ± SE of triplicates in a representative assay ($P < 0.0001$).

major sugar constituents from *B. adolescentis* M101-4 [29] and from *L. helveticus* var. *jugurti* [19]. Also, it is well known that various bacterial peptidoglycans induce antitumor activity through the modulation of immune systems. Tumor necrosis factor α and reactive nitrogen intermediates played a major role in the in vitro antitumor activity of mouse peritoneal exudates cells from mice stimulated with wall peptidoglycan from *B. infantis* [18]. Therefore, the direct inhibitory effect of BB-pol on tumor cell growth observed in the present study is rather exceptional for polysaccharide biomaterials extracted from various organisms. A partially purified cytotoxic substance from the culture supernatants of *L. casei* D-34 against three tumor cell lines – HeLa, HEP-2, and HFS-9 – was found to be proteinaceous in nature [20]. Recently, the cytoplasmic fraction from *Lactococcus lactis* ssp. *lactis* was reported to exert direct antiproliferation activity against the SNUC2A human colon cancer cell line by inducing S phase accumulation in SNUC2A cells [30], but the active components remain to be elucidated.

The present study shows that BB-pol is a novel polysaccharide with glucose, chiroinositol, and ribose as its major constituents, which inhibits the growth of colon cancer cell lines in vitro. We are currently analyzing general transcriptional responses of tumor cells following treatment with BB-pol to better understand the growth-inhibition mechanism of BB-pol.

Acknowledgement

This work was supported by the Korean Ministry of Science and Technology (Grant no. M1-0302-00-0098).

References

[1] Fuller, R. (1989) Probiotics in man and animals. J. Appl. Bacteriol. 66, 365–378.
[2] Goldin, B.R. (1998) Health benefits of probiotics. Br. J. Nutr. 80, S203–S207.
[3] Malhotra, S.L. (1977) Dietary factors in a study of colon cancer from Cancer Registry, with special reference to the role of saliva, milk and fermented milk products and vegetable fibre. Med. Hypotheses 3, 122–126.
[4] Sanders, M.E. (1998) Development of consumer probiotics for the US market. Br. J. Nutr. 80, S213–S218.
[5] Pool-Zobel, B.L., Neudecker, C., Domizlaff, I., Ji, S., Schillinger, U., Rumney, C., Moretti, M., Vilarini, I., Scassellati-Sforzolini, R. and Rowland, I. (1996) *Lactobacillus*- and *Bifidobacterium*-mediated antigenotoxicity in the colon of rats. Nutr. Cancer 26, 365–380.

[6] Arimochi, H., Kinouchi, T., Kataoka, K., Kuwahara, T. and Ohnishi, Y. (1997) Effect of intestinal bacteria on formation of azoxymethane-induced aberrant crypt foci in the rat colon. Biochem. Biophys. Res. Commun. 238, 753–757.
[7] Gallaher, D.D., Stallings, W.H., Blessing, L.L., Busta, F.F. and Brady, L.J. (1996) Probiotics, cecal microflora, and aberrant crypts in the rat colon. J. Nutr. 126, 1362–1371.
[8] Abdelali, H., Cassand, P., Soussotte, V., Daubeze, M., Bouley, C. and Narbonne, J.F. (1995) Effect of dairy products on initiation of precursor lesions of colon cancer in rats. Nutr. Cancer 24, 121–132.
[9] Onoue, M., Kado, S., Sakaitani, Y., Uchida, K. and Morotomi, M. (1997) Specific species of intestinal bacteria influence the induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats. Cancer Lett. 113, 179–186.
[10] Goldin, B.R., Gualtieri, L.J. and Moore, R.P. (1996) The effect of *Lactobacillus* GG on the initiation and promotion of DMH-induced intestinal tumors in the rat. Nutr. Cancer 25, 197–204.
[11] Goldin, B.R. and Gorbach, S.L. (1980) Effect of *Lactobacillus acidophilus* dietary supplements in 1, 2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats. J. Natl. Cancer Inst. 64, 263–265.
[12] Rowland, I.R., Rumney, C.J., Coutts, J.T. and Lievense, L.C. (1998) Effect of *Bifidobacterium longum* and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. Carcinogenesis 19, 281–285.
[13] Singh, J., Rivenson, A., Tomita, M., Shimamura, S., Ishibashi, N. and Reddy, B.S. (1997) *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. Carcinogenesis 18, 833–841.
[14] Kohwi, T., Imai, K., Tamura, A. and Hashimoto, Y. (1978) Antitumor effect of *Bifidobacterium infantis* in mice. Gann 69, 613–618.
[15] Kato, I., Kobayashi, S., Yokokura, T. and Mutai, M. (1981) Antitumor activity of *Lactobacillus casei* in mice. Gann 72, 517–523.
[16] Shahani, K.M. and Ayebo, A.D. (1980) Role of dietary lactobacilli in gastrointestinal microecology. Am. J. Clin. Nutr. 33, 2448–2457.
[17] Maclennan, R. and Jensen, O.M. (1977) Dietary fibre, transit time, fecal bacteria, steroids, and colon cancer in two Scandinavian populations. Lancet 30, 207–211.
[18] Sekine, K., Ohta, J., Onishi, M., Tatsuki, T., Shimokawa, Y., Toida, T., Kawashima, T. and Hashimoto, Y. (1995) Analysis of antitumor properties of effector cells stimulated with a cell wall preparation (WPG) of *Bifidobacterium infantis*. Biol. Pharm. Bull. 18, 148–153.
[19] Oda, M., Hasegawa, H., Komatsu, S., Kambe, M. and Tsuchiya, F. (1983) Anti-tumor polysaccharide from *Lactobacillus* sp. Agric. Biol. Chem. 47, 1623–1625.
[20] Manjunath, N. and Ranganathan, B. (1989) A cytotoxic substance produced by a wild culture of *Lactobacillus casei* D-34 against tumor cells. Indian J. Exp. Biol. 27, 141–145.
[21] Park, S.Y., Ji, G.E., Ko, Y.T., Jung, H.K., Ustunol, Z. and Pestka, J.J. (1999) Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. Int. J. Food Microbiol. 46, 231–241.
[22] Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M. and Strober, W. (1990) Trypan blue exclusion test of cell viability. In: Current Protocols in Immunology (John Wiley and Sons, eds.) vol. 2, p. A.3.3., Greene Publishing Associates and Wiley-Interscience, New York.
[23] Coconnier, M.-H., Bernet, M.F., Kerneis, S., Chauviere, G., Fourniat, J. and Servin, A.L. (1993) Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol. Lett. 110, 299–305.
[24] Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell 47, 323–330.
[25] Kim, I.H., Park, M.S. and Ji, G.E. (2003) Characterization of adhesion of *Bifidobacterium* sp. BGN-4 to human enterocyte-like Caco-2 cells. J. Microbiol. Biotechnol. 13, 276–281.
[26] Ebina, T. (2003) Activation of antitumor immunity by intratumor injection of biological preparations. Gan. To. Kagaku Ryoho 30, 1555–1558.
[27] Kado, H., Yoneta, Y., Takeo, S., Mitsui, M. and Watanabe, N. (1991) Studies on an enzymatically synthesized antitumor polysaccharide SPR-901. Chem. Pharm. Bull. 39, 1078–1079.
[28] Dinadayala, P., Lemassu, A., Granovski, P., Cerantola, S., Winter, N. and Daffe, M. (2004) Revisiting the structure of the anti-neoplastic glucans of *Mycobacterium bovis* bacille calmette-guomicron rin analysis of the different types of glucans produed by the vaccine substrains. J. Biol. Chem. 279, 12369–12378.
[29] Hosono, A., Lee, J., Ametani, A., Natsume, M., Hirayama, M., Adachi, T. and Kaminogawa, S. (1997) Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101-4. Biosci. Biotechnol. Biochem. 61, 312–316.
[30] Kim, J.Y., Woo, H.J., Kim, Y.S., Kim, K.H. and Lee, H.J. (2003) Cell cycle dysregulation induced by cytoplasm of *Lactococcus lactis* ssp. *lactis* in SNUC2A, a colon cancer cell line. Nutr. Cancer 46, 197–201.

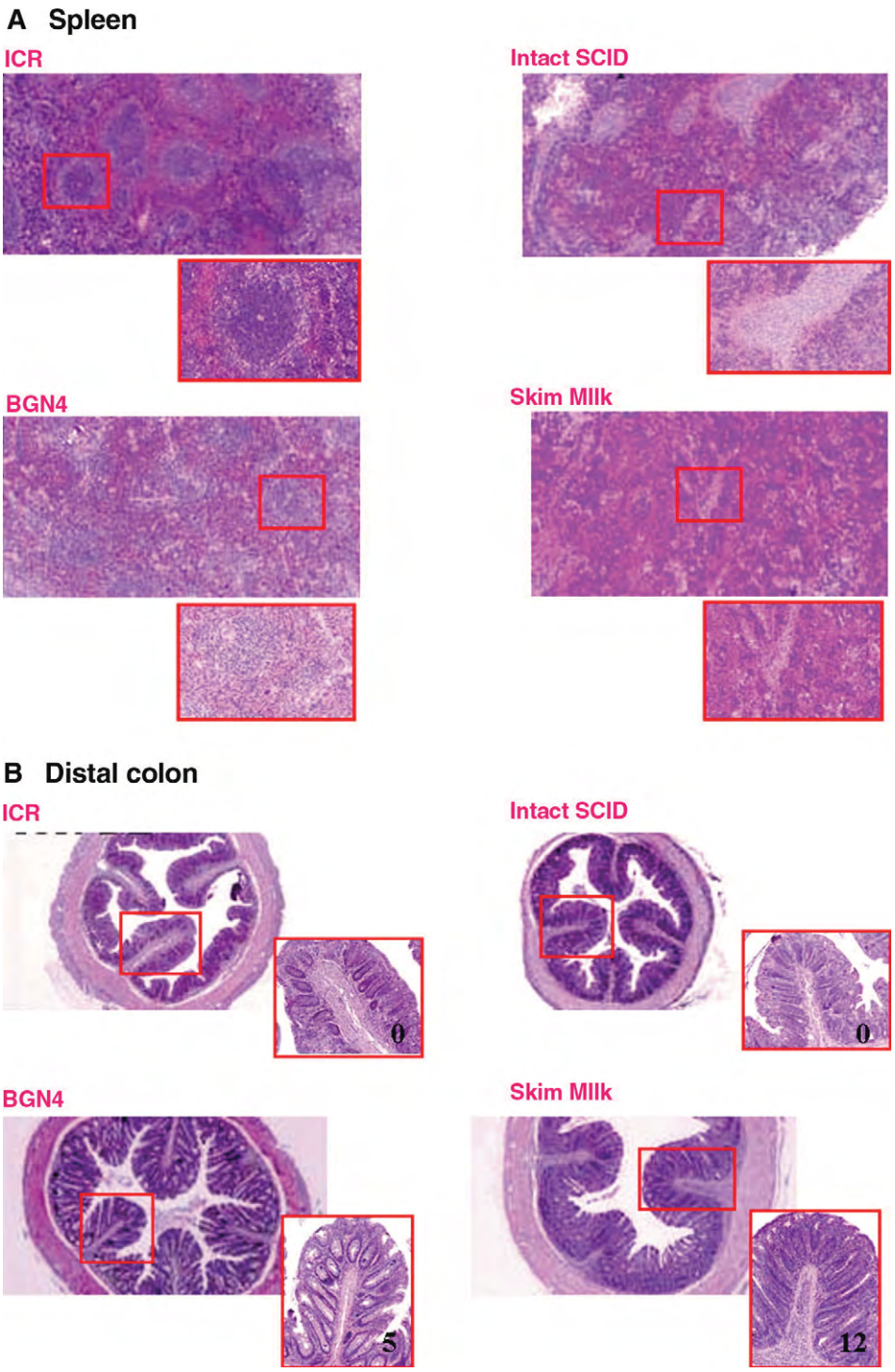

Figure 2 Prevention of lymphocyte accumulation in the spleen and large intestine by the feeding of BGN4. Spleen (A) and distal colon (B) were obtained from each group of mice and stained with H&E for histological analysis. The groups consisted of normal ICR (C.B-17/Icr) mice, intact SCID (C.B-17/Icr-Scid/Scid) mice, and $CD4^{+}$ $CD45RB^{high}$ T cell-transferred SCID (C.B-17/Icr-Scid/Scid) mice. They were fed with either BGN4 or skim milk. The written number in the small box indicates the mean of the histologic score of colonic inflammation. Original magnification is 25× and 100×.

score system from Corazza [27], the clinical degree of IBD was developed to a grade of 12 in the skim milk-fed mice while the clinical score was much lower in the BGN4-fed mice (a grade of 5). These results further provided convincing evidence for the preventive effects of BGN4 on the development of IBD.

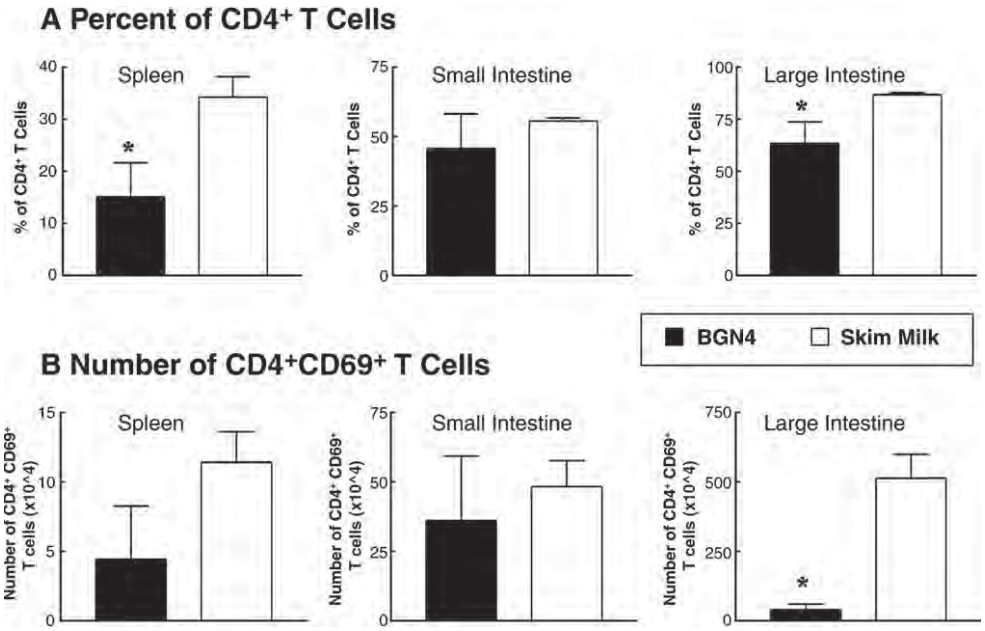

Figure 3 Distinct cellular appearances in the spleen and large intestine of BGN4-fed healthy or skim milk-fed IBD mice. After 4 weeks of adoptive transfer of the $CD4^+$ $CD45RB^{high}$ cells into SCID mice, the lymphocytes were isolated from the spleen and intestine. The percentages of the $CD4^+$ T cells (A) and the number of activated lymphocytes ($CD4^+$ $CD69^+$) (B) were measured in the groups of BGN4-fed mice and the skim milk-fed mice. Data are expressed as the mean value of SEM (*$p < 0.05$ versus skim milk-fed mice). Data were pooled from several independent experiments.

Observation of distinct cellular appearance in the spleen and large intestine between the BGN4-fed healthy mice and the skim milk-fed IBD mice

We next examined the appearance of the mononuclear cell in the spleen, small intestine, and large intestine of mice fed with BGN4 (Fig. 3). Flow cytometric analysis revealed that the percentage of $CD4^+$ T cells in the spleen and large intestine was reduced in the BGN4-treated group when compared with the skim milk-fed group with IBD (Fig. 3A). To investigate the activation stage of the $CD4^+$ T cells, that were isolated from each group, we measured the number of $CD4^+$ T cells expressing CD69, a well known T cell activation marker [19]. The number of $CD4^+$ T cells expressing CD69 was significantly decreased in the large intestine of the mice fed with BGN4 when compared with the skim milk-fed mice with IBD (Fig. 3B). These results indicate that BGN4 feeding results in the inhibition of expansion and activation of adoptively transferred pathological $CD4^+$ T cells in the spleen and large intestine of SCID mice.

Reduction of Th1-type cytokine production in the spleen and large intestine after the BGN4 feeding

The mononuclear cells from the spleen and large intestine of reconstituted SCID mice, fed with BGN4 or skim milk, were cultured for 48 h without any stimulation. The cytokine secretion patterns were then examined (Fig. 4). Mononuclear cells from the spleen and large intestine of the BGN4-fed group showed significantly reduced levels of IFN-γ and TNF-α in comparison with the skim milk-fed group. In contrast, Th2-type cytokines, IL-4, IL-5, and IL-10, showed identical levels between the skim milk-fed and BGN4-fed groups (Figs. 4A and B). These results further demonstrate that BGN4 feeding can influence the quality of the host immune system, specifically the inhibition of aberrant Th1-type cytokine synthesis leading to the inhibition of IBD development as observed in the adoptive transfer model.

BGN4 inhibition of IFN-γ and MCP-1 secretion in the co-culture system

To mimic the intestinal environment, we adopted a co-culture system using CMT93, epithelial cell lines derived from a mouse rectal carcinoma ($H\text{-}2^b$), and anti-CD3-activated lymphocytes from C57BL/6 ($H\text{-}2^b$), in order to assess the possible effects of BGN4 on the interaction between epithelial cells and T cells (Fig. 5). Interestingly, BGN4 inhibited Th1-type cytokine production, particularly the synthesis of IFN-γ and MCP-1, when compared to those of the co-culture without BGN4. In contrast, the secretion levels of inhibitory cytokine IL-10 were statistically not different, however, the level was slightly higher in the BGN4-fed group than the control group ($p = 0.1812$), whereas no significant changes were observed in the IL-6 secretion ($p = 0.3309$). Overall, these results further suggest that BGN4 possesses potent immunomodulatory functions on the homeostasis of the intestinal immune response and further provides protection from IBD development. However, we should point out that heat killed microorganism was used for

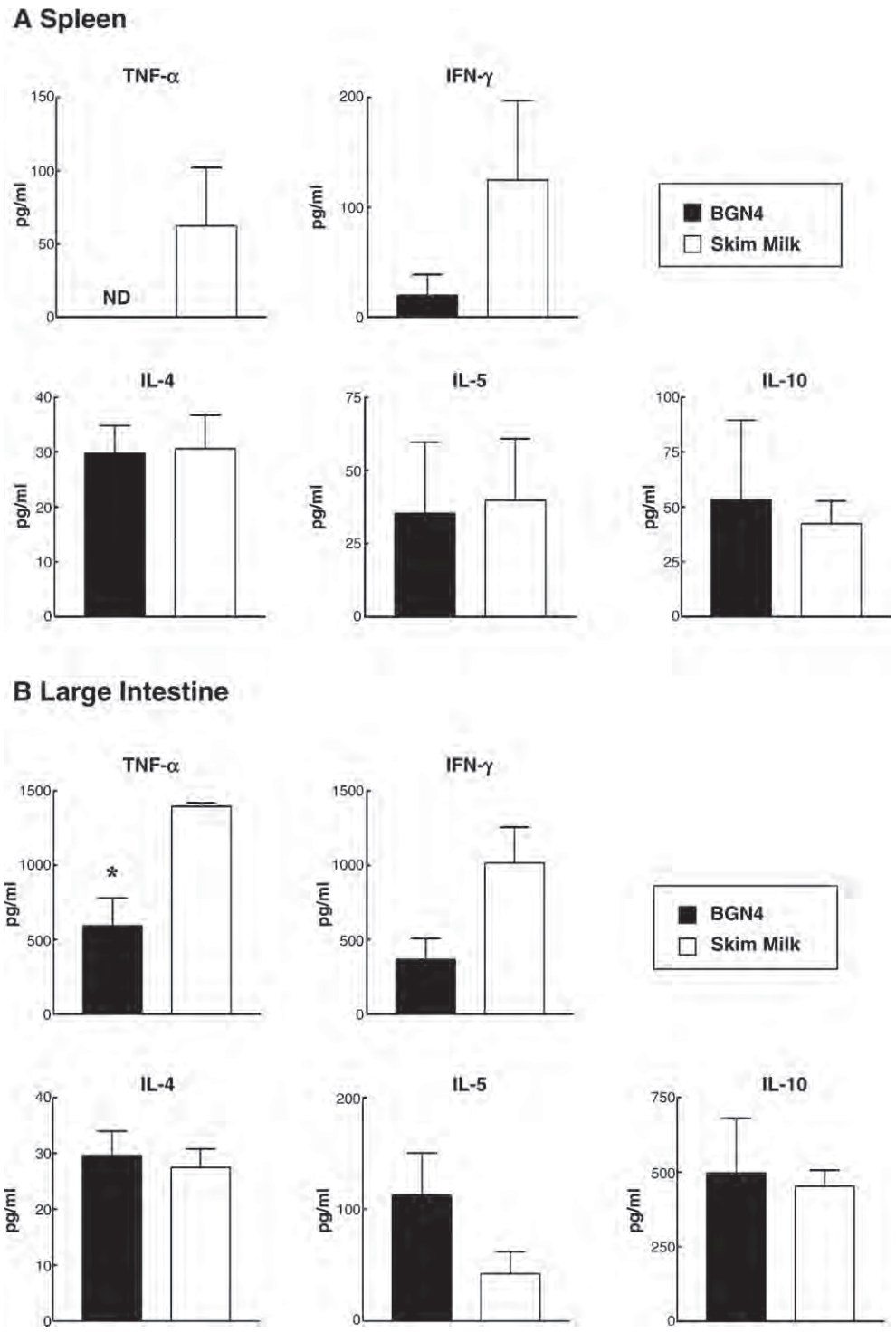

Figure 4 Inhibition of Th1 cytokine production in the BGN4-fed mice. Lymphocytes were isolated from the spleen (A) and large intestine (B) of the $CD4^+$ $CD45RB^{high}$ T cell-transferred mice fed with BGN4 or skim milk and then cultured for 48 h. Cytokine production in the culture supernatants was measured for the BGN4-fed mice and the skim milk-fed mice groups. Results are shown as mean value of SEM (*$p < 0.05$ versus skim milk-fed mice).

the *in vitro* study, which may not completely reflect *in vivo* situation. At least our *in vitro* study demonstrated that even heat killed BGN4 possessed immune modulation effect for the control of T cell cytokine responses.

Discussion

Probiotics, as preventive or therapeutic agents against IBD, have been shown to be an attractive and alternative

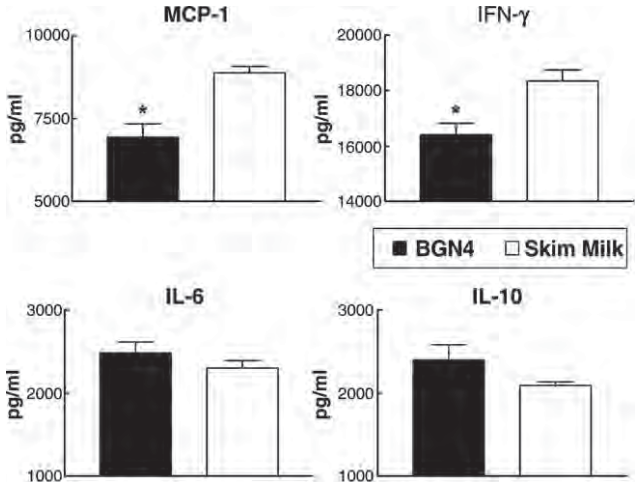

Figure 5 Analysis of cytokine production in the co-transwell culture with heat killed *B.* BGN4 (100 μg/ml) on the intestinal epithelial cell co-cultured with anti-CD3 mAb activated splenocytes. Following 48 h of incubation with BGN4, culture supernatants were harvested for cytokine detection using a CBA (BD) kit. Results are expressed as mean value of SEM (*$p < 0.05$ versus control).

approach for the attenuation of mucosal inflammation or systemic aberrant response [10,11]. It has been reported that several probiotic bifidobacterial strains and the products containing these bacteria possessed beneficial effects for controlling IBD in clinical trials [7,8]. Certain probiotic *Bifidobacterium* strains, isolated from human infant stools, produce antibacterial substances such as proteinaceous and lipophilic molecules that guard against one of the putative mucosal inflammation causing bacteria *Listeria monocytogenes* as well as the other enterovirulent *Salmonella* [28,29]. In the present study, we investigate an effect of *Bifidobacterium* BGN4 on the development of IBD using a *in vivo* animal model to directly observe the inhibitory mechanism of BGN4.

We employed the $CD4^+$ $CD45RB^{high}$ T cell transfer model of IBD to investigate the immunomodulatory role of *Bifidobacterium* BGN4 for the control of mucosal inflammation [17–19]. This is the first study that shows the preventive effects of *Bifidobacterium* on the development of IBD in the $CD4^+$ $CD45RB^{high}$ T cell reconstituted SCID model. Our present study demonstrates that the oral feeding of BGN4 inhibited the development of IBD (Fig. 1). This regulatory effect is derived from inhibiting the activation and accumulation of pathogenic lymphocytes in the large intestine (Figs. 2 and 3). Additionally, the production of aberrant Th1 response associated cytokines, IFN-γ and TNF-α, was reduced in the BGN4-fed group (Fig. 4). The fact that the BGN4 containing diet significantly reduced the production of TNF-α, which is one of responsible pathogenic cytokines in the $CD4^+$ $CD45RB^{high}$ T cell transfer IBD model [24], suggested that BGN4 could be an effective and alternative agent for the control of $CD4^+$ $CD45RB^{high}$ T cell-mediated IBD.

In our previous studies, it was shown that BGN4 strongly adhered to the intestinal epithelial cells [16], therefore, it was worthwhile to examine the possible influence of BGN4 on the cell to cell network of intestinal epithelial cells and T cells. We next adopted an *in vitro* co-culture system, consisting of confluent epithelial cell lines on a semi-porous membrane and basolaterally located activated T lymphocytes in order to mimic as much as possible an *in vivo* condition for the hyper-activated T cell-mediated intestinal environment. As shown in Fig. 5, addition of activated T cells onto the epithelial cell cultures resulted in increased IFN-γ and MCP-1 synthesis. Conversely, Th1 derived cytokine levels of IFN-γ and MCP-1, which are another major pathological contributing factor for the murine model of IBD [24,30], were dramatically decreased in the presence of BGN4 (Fig. 5). Although the possibility that BGN4 derived factors directly influence mucosal T cells cannot be eliminated, these results at least suggest that BGN4 may execute immunomodulatory effects through the intestinal epithelial cells to control the responses of proximal T lymphocytes in the intestinal epithelium and lamina propria regions.

When the degree of increased cell infiltration, an indication of inflammatory response, was examined, a noticeable change was noted in the spleen and large intestine of mice with IBD (Figs. 1B and 2). This change is consistent with previous findings [17–19]. On the other hand, these changes were not seen in the small intestine (data not shown). These results suggest the presence of a cross-talk system between the spleen and the large intestine. To this end, our previous study demonstrated that antigen-primed splenocytes preferentially migrated into the large intestine after repeated oral administration of the same antigen, which induced antigen-specific diarrhea [24]. When we examined the spleen and large intestine of mice fed with BGN4, there was no indication of an enlarged spleen with highly concentrated lymphocyte accumulation. Moreover, there was no amorphous pathological shape of the large intestine detected in comparison to the skim milk-fed mice with IBD (Figs. 1B and 2). The spleen and large intestine of the BGN4-fed mice were macro- and microscopically similar to the normal ICR mice absent any diseases (Figs. 1B and 2). Taken these facts together, one can suggest that the highly loaded BGN4 disciplined the behavior of the pathogenic lymphocytes and inhibited the abnormal hyper-responsiveness of the cross-talk system between the spleen and large intestine.

Another mode of BGN4 activity towards the prevention of IBD is the effect on bacteria. The growth of putative pathogenic bacteria (e.g. *Bacteroides* species) was inhibited by the growth competition with bifidobacteria [31]. It was also shown that antimicrobial properties of BGN4 may contribute to the development of IBD because *Bifidobacterium* has been proven to inhibit the growth of *Bacteroides* [31]. To address this possibility, we examined a composition of microflora in the intestine of mice fed with BGN4. Significant differences in the composition of putative pathogenic bacteria such as *Bacteroides* were not observed between the BGN4-fed normal and skim milk-fed IBD mice (data not shown). Although more extensive bacteriological analysis may be required to disprove the involvement of the antimicrobial activity of BGN4, the present data support the direct immunomodulatory effect of BGN4 on mucosal immunocompetent cells, including epithelial cells and T cells.

The inhibition of aberrant Th1 responses appears to be linked to the production of IL-10 since the cytokine has been

shown to inhibit inflammatory responses [12]. Thus, the presence of IL-10 is considered to be associated with the inhibition of Th1 cell-mediated IBD [32–34]. The results obtained by our *in vitro* study showed that IL-10 production was slightly increased by the feeding of BGN4, however not significantly different from the skim milk-fed mice with IBD (Fig. 4). A similar tendency was also noted in the co-culture system (Fig. 5). The involvement of recently reported glucocorticoid-induced TNFR family-related gene (GITR)$^+$ cells for the execution of inhibitory function, which was expressed in both of CD25$^+$ and CD25$^-$ regulatory T cells, was investigated [35,36]. However, the GITR expression level was similar in the splenic and large intestinal T cells, which were isolated from the skim milk- or BGN4-fed groups (data not shown). These results suggest that oral BGN4 feeding may not be directly involved in the induction of IL-10-producing and/or regulatory T cell-mediated inhibition of inflammatory responses.

One alternative possibility is that IL-10 might be produced from non-lymphocytes, such as dendritic cells (DCs) in the BGN4-fed mice, since it has been shown that DCs are another source of IL-10 for the control of Th1 responses [37]. Although we did not examine the effect of BGN4 on DC in this study, it is a subject of future study. Thus, it is still possible for BGN4 to induce regulatory function including IL-10 synthesis in controlling abnormal Th1 responses via DC. It was recently shown that nonpathogenic intestinal bacteria can induce DC to migrate into the epithelial layer for antigen sampling from the gut lumen [38]. Particularly in the gut, recruited DC by luminal antigen, including intestinal commensal bacteria, is important for transporting apoptotic intestinal epithelial cell to induce and maintain peripheral self-tolerance [39]. Therefore, a highly loaded and attached BGN4 on the intestinal epithelium may recruit regulatory type DC to the area, which will lead to the creation of inhibitory environments for unwanted hyper-responses to self and non-self antigens from the intestinal tract. We are currently examining this interesting mechanism.

It is also possible for BGN4 to induce other inhibitory pathways that attenuate inflammatory responses in the large intestine by the augmentation of other regulatory cytokines. In this regard, *Bifidobacterium* treatment has been shown to reduce Th1 cytokine production in IL-10 knockout mice [10]. This indicates the presence and activation of IL-10-independent inhibitory pathways by BGN4.

Although it was not our intent to investigate the effect of BGN4 on the innate immune system, we have to consider new perspectives that show progress, such as the role of toll-like receptors (TLR) on the intestinal epithelial cells and DC [40–43]. Commensal microorganisms are continuously interacting with the epithelial layer and presenting a number of innate immunity associated antigens via the receptors for pathogen-associated molecular patterns (PAMS). The interaction among TLR, PAMS, and commensal bacteria plays a role in controlling colonic epithelial cell turnover [44] and attenuation/trafficking of apical membrane receptors such as TLR2 and TLR4 [45]. Since BGN4 has been shown to adhere strongly to the intestinal epithelial cells [16], a connection might be made for the BGN4 derived specific protective immunomodulatory activity via constitutive interaction with TLRs presiding in the colonic epithelium [40]. This pathway seems to be a more plausible explanation of the immunomodulatory effects of BGN4 since a separate study showed that BGN4 was not effective against dextran sodium sulfate (DSS)-induced IBD (Fig. S1), where DSS directly and physically injures epithelial cells [4]. In our present results, IFN-γ and MCP-1 production from the basolateral chamber containing T lymphocytes was decreased when the epithelial cells were exposed to BGN4 (Fig. 5). However, this inhibitory effect was not observed when lymphocytes were directly exposed to BGN4. A recent report also indicated that probiotic DNA has anti-inflammatory effects on IBD through TLR9 signaling [46]. Therefore, we consider the suppression of IBD to be revealed via the relationship between BGN4 and the epithelial cells in the intestinal immune system.

Acknowledgments

This work was supported by grants from the Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation (JST); the Ministry of Education, Science, Sports, and Culture; the Ministry of Health and Labor in Japan; and Uehara Memorial Foundation in Japan. This work was also supported by a grant from the Korean Ministry of Science and Technology of Korea (Grant no. M1-0302-00-0098).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clim.2006.11.005.

References

[1] G. Bouma, W. Strober, The immunological and genetic basis of inflammatory bowel disease, Nat. Rev., Immunol. 3 (7) (2003) 521–533.
[2] C.O. Elson, Experimental models of intestinal inflammation. New insights into mechanisms of mucosal homeostasis, in: P.L. Orga, et al., (Eds.), Handbook of Mucosal Immunology, Academic Press, San Diego, 1999, p. 1007–1024.
[3] C. Fiocchi, Inflammatory bowel disease: etiology and pathogenesis, Gastroenterology 115 (1) (1998) 182–205.
[4] R.B. Sartor, Microbial factors in the pathogenesis of Crohn's disease, ulcerative colitis and experimental intestinal inflammation, in: J.B. Kirsner, R.G. Shorter (Eds.), Inflammatory Bowel Disease, Williams and Wilkins, Baltimore, 1995, p. 96–104.
[5] R.K. Linskens, et al., The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics, Scand. J. Gastroenterol., Suppl. 234 (2001) 29–40.
[6] G. Bamias, et al., Down-regulation of intestinal lymphocyte activation and Th1 cytokine production by antibiotic therapy in a murine model of Crohn's disease, J. Immunol. 169 (9) (2002) 5308–5314.
[7] A.C. Ouwehand, S. Salminen, E. Isolauri, Probiotics: an overview of beneficial effects, Antonie Van Leeuwenhoek 82 (1–4) (2002) 279–289.
[8] C. Shortt, The probiotic century: historical and current perspectives, Trends Food Sci. Technol. 10 (12) (1999) 411–417.
[9] N. Borruel, et al., Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria, Gut 51 (5) (2002) 659–664.
[10] J. McCarthy, et al., Double blind, placebo controlled trial of two

probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance, Gut 52 (7) (2003) 975—980.
[11] K.L. Madsen, et al., *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice, Gastroenterology 116 (5) (1999) 1107—1114.
[12] L. Steidler, et al., Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10, Science 289 (5483) (2000) 1325—1352.
[13] A. Bezkorovanyl, Ecology of bifidobacteria, in: A. Bezkorovainy, R. Miller-Catchpole (Eds.), Biochemistry and Physiology of Bifidobacteria, CRC Press, Florida, 1989, p. 29—72.
[14] J.E. Teitelbaum, W.A. Walker, Nutritional impact of pre- and probiotics as protective gastrointestinal organisms, Annu. Rev. Nutr. 22 (2002) 107—138.
[15] L. Steidler, Microbiological and immunological strategies for treatment of inflammatory bowel disease, Microbes Infect. 3 (13) (2001) 1157—1166.
[16] I.H. Kim, M.S. Park, G.E. Ji, Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like caco-2 cells, J. Microbiol. Biotechnol. 13 (2) (2003) 276—281.
[17] P.J. Morrissey, et al., $CD4^+$ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified $CD4^+$ T cells, J. Exp. Med. 178 (1) (1993) 237—244.
[18] F. Powrie, et al., Regulatory interactions between $CD45RB^{high}$ and $CD45RB^{low}$ $CD4^+$ T cells are important for the balance between protective and pathogenic cell-mediated immunity, J. Exp. Med. 179 (2) (1994) 589—600.
[19] R. Aranda, et al., Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of $CD4^+$, $CD45RB^{high}$ T cells to SCID recipients, J. Immunol. 158 (7) (1997) 3464—3473.
[20] S.J. Ott, et al., Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease, Gut 53 (5) (2004) 685—693.
[21] Y.J. Choi, et al., Growth and beta-glucosidase activity of *Bifidobacterium*, J. Microbiol. Biotechnol. 6 (4) (1996) 255—259.
[22] D. Kishi, et al., Alteration of V β usage and cytokine production of $CD4^+$ TCR $\beta\beta$ homodimer T cells by elimination of *Bacteroides vulgatus* prevents colitis in TCR α-chain-deficient mice, J. Immunol. 165 (10) (2000) 5891—5899.
[23] I. Takahashi, H. Kiyono, S. Hamada, $CD4^+$ T-cell population mediates development of inflammatory bowel disease in T-cell receptor α chain-deficient mice, Gastroenterology 112 (6) (1997) 1876—1886.
[24] M.N. Kweon, et al., Development of antigen induced colitis in SCID mice reconstituted with spleen derived memory type CD4(+) CD45RB(+) T cells, Gut 50 (3) (2002) 299—306.
[25] A. Parlesak, et al., Modulation of cytokine release by differentiated CACO-2 cells in a compartmentalized coculture model with mononuclear leucocytes and nonpathogenic bacteria, Scand. J. Immunol. 60 (5) (2004) 477—485.
[26] A.K. Abbas, A.H. Lichtman, J.S. Pober, Cell and tissue of the immune system, in: W. Shmitt (Ed.), Cellular and Molecular Immunology, WB Saunders, Philadelphia, 1997, p. 15—33.
[27] N. Corazza, et al., Nonlymphocyte-derived tumor necrosis factor is required for induction of colitis in recombination activating gene (RAG)2(−/−) mice upon transfer of CD4(+) CD45RB(hi) T cells, J. Exp. Med. 190 (10) (1999) 1479—1492.
[28] V. Lievin, et al., *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity, Gut 47 (5) (2000) 646—652.
[29] R. Toure, et al., Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes*, J. Appl. Microbiol. 95 (5) (2003) 1058—1069.
[30] H. Scheerens, et al., Characterization of chemokines and chemokine receptors in two murine models of inflammatory bowel disease: IL-10−/− mice and Rag-2−/− mice reconstituted with $CD4^+CD45RB^{high}$ T cells, Eur. J. Immunol. 31 (5) (2001) 1465—1474.
[31] T. Shiba, et al., The suppressive effect of bifidobacteria on *Bacteroides vulgatus*, a putative pathogenic microbe in inflammatory bowel disease, Microbiol. Immunol. 47 (6) (2003) 371—378.
[32] B.E. Sands, Therapy of inflammatory bowel disease, Gastroenterology 118 (2 Suppl. 1) (2000) 68—82.
[33] C. Asseman, S. Read, F. Powrie, Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by $CD4^+$ regulatory T cells and IL-10, J. Immunol. 171 (2) (2003) 971—978.
[34] M.W. Leach, et al., The role of IL-10 in inflammatory bowel disease: "of mice and men", Toxicol. Pathol. 27 (1) (1999) 123—133.
[35] K. Uraushihara, et al., Regulation of murine inflammatory bowel disease by $CD25^+$ and $CD25^-$ $CD4^+$ glucocorticoid-induced TNF receptor family-related gene$^+$ regulatory T cells, J. Immunol. 171 (2) (2003) 708—716.
[36] F. Kanamaru, et al., Costimulation via glucocorticoid-induced TNF receptor in both conventional and $CD25^+$ regulatory $CD4^+$ T cells, J. Immunol. 172 (12) (2004) 7306—7314.
[37] R.J. Rigby, et al., Production of interleukin (IL)-10 and IL-12 by murine colonic dendritic cells in response to microbial stimuli, Clin. Exp. Immunol. 139 (2) (2005) 245—256.
[38] M. Rescigno, et al., Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria, Nat. Immunol. 2 (4) (2001) 361—367.
[39] F.P. Huang, A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes, J. Exp. Med. 191 (3) (2000) 435—444.
[40] S. Rakoff-Nahoum, et al., Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis, Cell 118 (2) (2004) 229—241.
[41] A.J. Macpherson, T. Uhr, Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria, Science 303 (5664) (2004) 1662—1665.
[42] H.R. Christensen, H. Frokiar, J.J. Pestka, Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells, J. Immunol. 168 (1) (2002) 171—178.
[43] M. Drakes, T. Blanchard, S. Czinn, Bacterial probiotic modulation of dendritic cells, Infect. Immun. 72 (6) (2004) 3299—3309.
[44] J.B. Seidelin, Colonic epithelial cell turnover: possible implications for ulcerative colitis and cancer initiation, Scand. J. Gastroenterol. 39 (3) (2004) 201—211.
[45] E. Cario, et al., Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium, Am. J. Pathol. 160 (1) (2002) 165—173.
[46] D. Rachmilewitz, et al., Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis, Gastroenterology 126 (2) (2004) 520—528.

J. Microbiol. Biotechnol. (2005), **15**(4), –

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of *Bifidobacterium* Cell Fractions on IL-6 Production in RAW 264.7 Macrophage Cells

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Received: September 13, 2004
Accepted: January 18, 2005

Abstract *Bifidobacterium* has been previously shown to potentiate immune function, which was mediated through the stimulation of cytokine production by macrophage. This study was performed to further characterize the effective component of *Bifidobacterium* by measuring the level of interleukin (IL)-6 cytokine using the RAW 264.7 murine cell line as a macrophage model. RAW 264.7 cells were cultured for 24 h in the presence of whole cells (WCs), cell walls (CWs), and cell free extracts (CFEs) from various strains of *Bifidobacterium* and other lactic acid bacteria at various concentrations. The most effective component was different depending on the strains and the concentrations used. When tested with each cell fraction from *Bifidobacterium* sp. BGN4, heat treatment of the cell fractions lowered the production of IL-6. Synergistic effect was obtained especially when CWs and CFEs were combined. Sonicated WCs stimulated IL-6 production more than intact WCs. The *in vitro* approaches employed here should be useful in further characterization of the effects of *Bifidobacterium* on gastrointestinal and systemic immunity.

Key words: *Bifidobacterium*, IL-6, macrophage, cell fractions

Bifidobacterium is predominant in the lumen of the large intestine. In breast-fed infants, *Bifidobacterium* comprises more than 90% of the gut bacterial population [19], however, their numbers gradually decrease over the life time of the host. *Bifidobacterium* spp. is used in commercial fermented dairy products and has been suggested to exert health promoting effects on the host by maintaining intestinal microflora balances, improving lactose tolerance, preventing inflammatory bowel disease, and aiding anticarcinogenic activity [10, 11, 13]. In addition, Hattori *et al.* [8] reported that the administration of *Bifidobacterium* for 1 month to children with atopic dermatitis showed significant improvement of allergic symptoms.

Other beneficial effects of the intake of *Bifidobacterium* are reported to include reinforcement of immune functions [28]. It has been shown that *Bifidobacterium* enhances several immune functions, namely macrophage and lymphocyte activation [7, 25], cytokine secretion [16], mitogenic response in spleen and Peyer's patches [9, 12, 28]. Such stimulation of the immune response by *Bifidobacterium* has been proposed to enhance resistance to infection by pathogenic organisms [24] and potentially prevent cancer [25, 26]. Cell components of *Bifidobacterium* which function as immunomodifiers of the host reportedly include peptidoglycan, intra and extracellular polysaccharide products, cell free extracts (CFEs), and cell walls (CWs) [6, 7, 9, 20, 25, 26]. However, at the present time, there is not yet a clear understanding of the molecular and cellular basis for immunomodulation by *Bifidobacterium.* To utilize a potential of the *Bifidobacterium* for immunomodulation of the host, better understanding of the quantitative and qualitative assessment of the immunomodulatory effect of the *Bifidobacterium* is needed.

Miettinen *et al.* [18] measured production of tumor necrosis factor (TNF)-alpha, interleukin (IL)-6, and IL-10 from human peripheral blood mononuclear cells after stimulation with live or glutaraldehyde-fixed bacteria and suggested that lactic acid bacteria can stimulate nonspecific immunity. Using macrophage as a model system, we earlier showed that the modulatory effect of the *Bifidobacterium* on IL-6 production was strain- and dose-dependent. In the present study, we further characterized the different cell components and their processing on the immunostimulation in the RAW 264.7 murine macrophage model by measuring IL-6 production as a representative macrophage cytokine.

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The results showed that the stimulatory activity of the *Bifidobacterium* cell fractions differed, depending on the strains and the concentrations used and also processing conditions of the cell components. Our study suggests that better understanding of the effective component and the immunological state of the host is necessary to achieve a desirable immunological balance for the host.

MATERIALS AND METHODS

Bifidobacterium Cultures

B. adolescentis 15703, *B. longum* 15707, *B. infantis* 15697, and *B. breve* 15700 were obtained from American Type Culture Collection (Rockville, MD). The identification and experimental use of *Bifidobacterium* sp. BGN4, *Bifidobacterium* sp. JH-2, *Bifidobacterium* sp. MS-1, *Bifidobacterium* sp. SJ342, *Bifidobacterium* sp. UN-4, and other lactic bacteria were reported before [3, 22]. All strains were cultured and subcultured anaerobically in MRS broth (Difco, Detroit, MI, U.S.A.) containing 5% (wt/vol) lactose (MRSL) at 37°C until late log phase [15, 21]. Cells were collected by centrifugation at 1,000 ×*g* and 4°C for 15 min and washed twice with PBS, followed by final washing with distilled water [14]. They were dried by Speed-Vac (Instruments, INC., N.Y., U.S.A.) and resuspended in Hanks' buffered salt solution (Sigma Chemical Co., St. Louis, MO, U.S.A.) to desired bacterial concentration on a dry weight basis. For introduction into tissue culture, *Bifidobacterium* was ordinarily killed by heating at 100°C for 20 min. Heat-killed cultures were aliquoted and stored at −80°C until used.

Preparation of Cell Fractions

For the preparation of the cell fractions, cultured bacterial cells were collected by centrifugation at 2,000 ×*g* for 20 min (Hanil MF-80, Inchun, Korea) and washed twice with autoclaved PBS, followed by final washing with autoclaved distilled water. For the preparation of CWs and CFEs, washed WCs were disrupted by French Pressure Cell Press (Spectronic, Rochester, N.Y., U.S.A.). The WCs were removed from the suspension by centrifugation at 2,000 ×*g* for 20 min. CWs were sedimented by centrifugation at 15,000 ×*g* and 4°C for 45 min (Hanil Micro 17R+, Inchon, Korea) and the supernatant was used as CFEs. Each fraction was freeze dried and resuspended with DMEM to the desired concentration on a dry weight basis. Suspended bacterial fractions were stored at −20°C until used.

Chemicals and Reagents

IL-6, purified antibodies to IL-6 antibodies (rat anti-mouse), and biotinylated rat anti- or IL-6 were obtained from PharMingen (San Diego, CA, U.S.A.). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Chagrin Falls, IL, U.S.A.).

Cell Culture

The mouse macrophage cell line RAW264.7 (American Type Tissue Collection) was grown in DMEM supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 1% (v/v) NCTC-135, streptomycin (100 μg/ml) and penicillin (100 U/ml). All cultures were carried out at 37°C in a humidified atmosphere with 5% CO_2 [29]. Cell number and viability were assessed by trypan blue dye exclusion [27] on a Neubauer hemacytometer (American Optical, Buffalo, N.Y., U.S.A.). Cells were grown to confluency in sterile tissue culture dishes and gently detached by repeated pipetting. For experiments, cells were cultured in triplicate at a density of 5×10^5 cells/ml in 96-well flat-bottomed tissue culture plates (Costar, Cambridge MA, U.S.A.). Cultures containing bacterial cell fractions were incubated for various time intervals and analyzed for IL-6. Heat treatment of the fractions were ordinarily done at 100°C for 20 min to sterilize bacterial contaminants. When unheated fractions were assessed, they were treated at 10 cm below ultraviolet light (Sankyo Germicidal UV Lamp G40TO, Japan) for 30 min.

IL-6 Quantitation

Production of IL-6 was quantitated by ELISA using modification of the procedure of Dong *et al.* [4]. Briefly, microtiter strip wells (Immunolon IV Removawell; Dynatech Laboratories Inc., Chantilly, VG) were coated overnight at 4°C with 50 μl of 1 μg/ml purified antibodies to IL-6 antibodies (rat anti-mouse) in 0.1 M sodium bicarbonate buffer (pH 8.2). Wells were incubated with 300 μl of 3% (v/v) bovine serum albumin (BSA) in 0.01 M PBS (pH 7.2), containing 0.2% (v/v) Tween 20 (PBST), at 37°C for 30 min to block nonspecific protein binding. Standard recombinant murine IL-6 and samples, diluted in 10% (v/v) FBS RPMI-1640, were added in 50 μl aliquots to appropriate wells and incubated at 37°C for 1 h. After washing four times with PBST, biotinylated rat anti-mouse IL-6 antibodies were diluted in BSA-PBST to 1 μg/ml and 1.5 μg/ml, respectively, and 50 μl each were added and incubated at room temperature for 1 h. Plates were washed six times and incubated with 50 μl of streptavidin-horseradish peroxidase conjugate (1.5 μg/ml in BSA-PBST) at room temperature for 1 h. After washing eight times, bound peroxidase conjugate was detected by adding 100 μl/well solution of substrate consisting of 25 ml of 0.1 M citric-phosphate buffer (pH 5.5), 0.1 mg/ml TMB, and 100 μl of 1% H_2O_2. An equal volume of 6 N H_2SO_4 was added to stop the reaction. The plates were read at 450 nm on a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA, U.S.A.). IL-6 were quantitated using

Table 1. Effect of *Bifidobacterium* and *Lactobacillus* cell fractions on IL-6 production by RAW 264.7 cells.

Strain	Treatment	IL-6 concentration (ng/ml)								
		WC (μg/ml)			CW (μg/ml)			CFE (μg/ml)		
		0.5	2.5	12.5	0.5	2.5	12.5	0.5	2.5	12.5
B. adolescentis ATCC 15703		ND	ND	1.5±0.3	ND	10.4±0.3	23.3±1.1	ND	1.7±0.2	4.2±0.3
B. breve ATCC 15700		ND	1.7±0.3	5.0±0.4	ND	2.1±0.2	11.3±0.2	ND	2.5±0.1	5.8±0.1
B. infantis ATCC 15697		ND	ND	4.5±0.7	ND	10.8±0.5	21.7±1.0	ND	ND	10.0±0.5
B. longum ATCC 15707		ND	ND	14.1±0.3	ND	1.8±0.2	22.5±0.9	ND	10.8±0.4	18.8±0.7
Bifidobacterium sp. BGN4		ND	1.5±0.3	9.3±0.5	ND	2.7±0.3	10.8±1.0	ND	5.2±0.1	15.8±0.4
Bifidobacterium sp. JH2		ND	ND	3.3±0.2	ND	ND	11.7±0.5	ND	8.3±0.9	12.1±0.3
Bifidobacterium sp. MS1		ND	ND	1.7±0.3	ND	3.0±0.2	8.2±0.3	ND	2.9±0.2	4.8±0.2
Bifidobacterium sp. SJ32		ND	ND	1.5±0.1	ND	8.3±0.4	14.8±0.2	ND	4.2±0.4	9.0±0.2
Bifidobacterium sp. UN4		ND	12.8±0.9	10.8±0.4	ND	9.2±0.2	12.7±0.1	ND	2.5±0.2	12.3±1.0
Lactobacillus sp. L1		ND	ND	ND	ND	ND	1.7±0.2	ND	ND	ND
Lactobacillus sp. L2		ND	ND	ND	ND	ND	2.5±0.3	ND	1.6±0.2	5.8±0.5
Lactobacillus sp. L4		ND	ND	ND	ND	ND	2.6±0.4	ND	ND	1.8±0.3
Lactobacillus sp. L7		ND	ND	ND	ND	ND	2.8±0.4	ND	ND	ND

Vmax Software (Molecular Devices). Triplicate samples were used throughout the experiments.

RESULTS

Effect of Cell Fractions of Various *Bifidobacterium* on IL-6 Production by RAW 264.7 Cells

To assess the effects of the cell fractions from various *Bifidobacterium* strains on the production of IL-6, macrophages RAW 264.7 cells (5×10^5 cells/ml) were incubated in the presence of 0.5, 2.5, and 12.5 μg/ml bacterial cell fractions. To prevent contamination, all bacterial fractions were routinely preheated at 100°C for 20 min. The results for IL-6 production are shown in Table 1. The degree of IL-6 production differed depending on the strains and the concentrations of the cell fractions used. All three fractions from MS-1 strain showed the low level of IL-6 production, compared with the other strains. CWs from most of the strains such as *B. adolescentis* 15703, *B. longum* 15707, *B. infantis* 15697, *B. breve* 15700, *Bifidobacterium* sp. MS-1, and *Bifidobacterium* sp. SJ-32 showed stronger activity than WCs or CFEs, whereas CFEs from BGN-4 showed higher IL-6 production than WCs or CWs. The production of IL-6 tended to increase in a dose dependent manner up to 12.5 μg/ml of all three fractions from most of the strains. For comparison, cell fractions from four strains of lactic acid bacteria were assessed. Fractions from *Lactobacillus* strains showed much lower activity than those from *Bifidobacterium*.

Effect of Heat Treatment and Combination of the Cell Fractions on IL-6 Production

To assess the effects of combination of the cell fractions, the mixed fractions from *Bifidobacterium* sp. BGN4 were added to RAW264.7 cells. The combinations were as follows; WCs plus CWs, CWs plus CFEs, and WCs plus

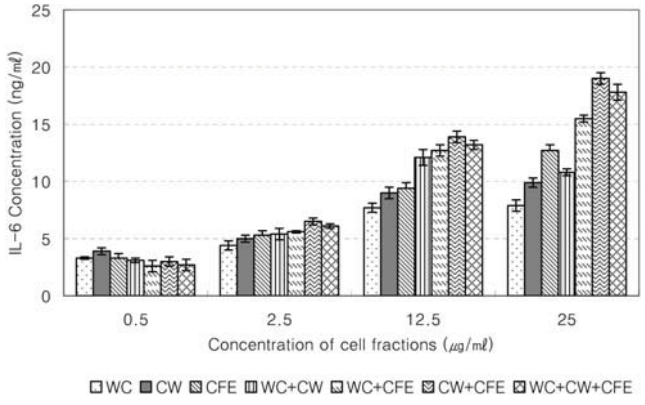

Fig. 1. Effect of combination of BGN cell fractions on IL-6 by RAW 264.7 cells.

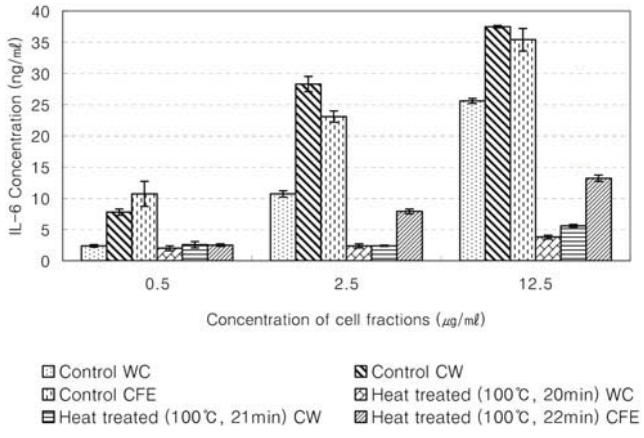

Fig. 2. Effect of heat treatyment of BGN4 cell fractions on IL-6 production by RAW 264.7 cells.

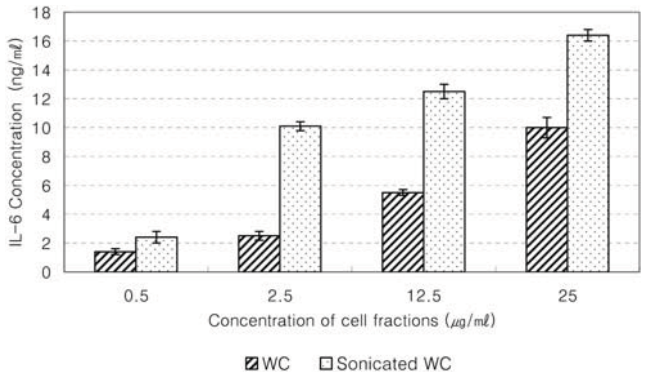

Fig. 3.

CFEs. Their final concentrations were 0, 0.5, 12.5 and 25 μg/ml, and each combination contained equal amount of respective fractions. The results are shown in Fig. 1. A synergistic effect was observed when mixed cell fractions were used on the macrophage stimulation. Combination of CWs and CFEs showed the highest stimulation. In addition, sonicated WCs which was a mixture of different cell fractions showed higher stimulation than WCs (Fig. 2). When heated and unheated fractions were compared, unheated fractions had much stronger stimulatory activity than heated fraction (Fig. 3). The experimental values of IL-6 production under the same treatment conditions or the control values at the base line slightly differed from experiment to experiment, but showed similar tendency.

DISCUSSION

Bifidobacterium and other lactic acid bacteria have been previously shown to stimulate immune function [6, 12, 17]. Furthermore, *Bifidobacterium* and other lactic acid bacteria can improve antitumor activity of the host [5, 23, 26]. It has been suggested that this activity may be due to their ability to stimulate macrophages and T cells [7, 25]. Sekine *et al.* [26] have proposed critical roles of cytokines played in the antitumor promoting properties of *B. infantis*, however, the mechanisms by which such bacteria modulate the immune response remains unclear. There is extensive evidence that cytokines play pivotal roles in host defense, inflammatory responses, and autoimmune disease [1, 2].

Park *et al.* [22] showed that both human and commercial *Bifidobacterium* strains can stimulate hydrogen peroxide, nitric oxide, TNF-alpha, and IL-6 productions, and this effect was dependent on dose and strain. In the present study, this phenomenon was further confirmed by the fact that the stimulating activity of each fraction was different, depending on the strain and the dose. This may explain the reason of why different authors reported different cell components of *Bifidobacterium* as immunomodifiers of the host, including peptidoglycan, intra- and extra-cellular polysaccharide products, CFEs, and CWs [6, 7, 9, 20, 22, 25, 26]. Park *et al.* [22] suggested that even morphology and composition of the *Bifidobacterium* cell might play an important role in macrophage activation and showed that the morphology of the *Bifidobacterium* varied depending on the growth temperature, thus suggesting that even the same strain may show varying stimulatory effect on macrophage depending on the growth conditions or environmental conditions to which *Bifidobacterium* was exposed. Even further, processing conditions of the active materials affect the IL-6 production by different degrees. In the present study, heat treatment decreased the production of IL-6, and combination of cell fractions had a synergistic effect on the production of IL-6. From the point of health, a balanced immune state is desired, whereas too much enhancement or too much suppression leads to the state of various immune diseases. Therefore, the control of activity of the immune cells is very important. Although these organisms are incorporated into dairy foods or pharmaceuticals, the benefits of these products have not typically been backed by clinical and/or microbiological studies. The present study provides some insight into how and what to consider when designing the application of the probiotic *Bifidobacterium* in order to provide health benefits from the immunological standpoint. However, further studies are necessary.

In conclusion, the results reported here showed that various sources of *Bifidobacterium* increased the secretion of several mediators by macrophage, thereby potentially modulating the host immune response. The results also showed that this stimulatory capacity was affected by dose and strain of the *Bifidobacterium* strain. The *in vitro* approaches employed here should be useful in future characterization of understanding mechanism of the effects of *Bifidobacterium* on gastrointestinal immunity and exploitation of possible enhancement of the health benefits using *Bifidobacterium* products.

Acknowledgment

This work was supported by the Korean Ministry of Science and Technology (M1-0302-00-0098).

REFERENCES

1. Abbas, A. K., A. H. Lichtman, and J. S. Pobe. 1994. Cytokines, p. 240. *In* Cellular and Molecular Immunology. 2nd ed. W. B. Saunders Co., Philadelphia, PA.
2. Akira, S., T. Taga, and T. Kishimoto. 1993. Interleukin-6 in biology and medicine. *Adv. Immunol.* **54:** 1–78.

3. Choi, Y. J., C. J. Kim, S. Y. Park, Y. T. Ko, H. K. Jeong, and G. E. Ji. 1996. Growth and beta-glucosidase activity of *Bifidobacterium*. *J. Microbiol. Biotechnol*. **6:** 255–259.
4. Dong, W., J. I. Azcona-Olivera, K. H. Brooks, J. E. Linz, and J. J. Pestka. 1994. Elevated gene expression and production of interleukins 2, 4, 5, and 6 during exposure to vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. *Toxicol. Appl. Pharmacol*. **127:** 282–290.
5. Fernandes, C. F. and K. M. Shahani. 1990. Anticarcinogenic and immunological properties of dietary lactobacilli. *J. Food Prot*. **53:** 704–710.
6. Gomez, E., M. M. Melgar, G. P. Silva, A. Portoles, and I. Gil. 1988. Exocellular products from *Bifidobacterium adolescentis* as immunomodifiers in the lymphoproliferative responses of mouse splenocytes. *FEMS Microbiol. Lett*. **56:** 47–52.
7. Hatcher, G. E. and R. S. Lambrecht. 1993. Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid-producing bacteria. *J. Dairy Sci.* **76:** 2485–2492.
8. Hattori, K., A. Yamamoto, M. Sasai, S. Taniuchi, T. Kojima, Y. Kobayashi, H. Iwamoto, K. Namba, and T. Yaeshima. 2003. Effects of administration of bifidobacteria on fecal microflora and clinical symptoms in infants with atopic dermatitis**.** *Arerugi*. **252:** 20–30.
9. Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S. Kaminogawa. 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterim adolescentis* M 101-4. *Biosci. Biotech. Biochem*. **61:** 312–316.
10. Hughes, D. B. and D. G. Hoover. 1991. *Bifidobacterium*: Their potential for use in American dairy products. *Food Technol.* **45:** 74–83.
11. Ishikawa, H, I. Akedo, Y. Umesaki, R. Tanaka, A. Imaoka, and T. Otani. 2003. Randomized controlled trial of the effect of *Bifidobacterium*-fermented milk on ulcerative colitis. *J. Am. Coll. Nutr.* **22:** 256–263
12. Kado-Oka, Y., S. Fujiwara, and T. Hirota. 1991. Effects of *Bifidobacterium* cells on mitogenic response of splenocytes and several functions of phagocytes. *Milchwissenshaft* **46:** 626–630.
13. Kanauchi, O., K. Mitsuyama, Y. Araki, and A. Andoh. 2003. Modification of intestinal flora in the treatment of inflammatory bowel disease. *Curr Pharm Des.* **9:** 336–346.
14. Kim, H. J., J. H. Kim, J. H. Son, H. J. Seo, S. J. Park, M. S. Paek, and S. K. Kim. 2004. Characterization of bacteriocin produced by *Lactobacillus bulgaricus*. *J. Microbiol. Biotechnol.* **14:** 503–508.
15. Kim, I. H., M. S. Park, and G. E. Ji. 2003. Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 cells. *J. Microbiol. Biotechnol.* **13:** 276–281.
16. Lammers, K. M., P. Brigidi, B. Vitali, P. Gionchetti, F. Rizzello, E. Caramelli, D. Matteuzzi, and M. Campieri. 2003. Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. *FEMS Immunol. Med. Microbiol*. **38:** 165–172.
17. Lee, J., A. Ametani, A. Enomoto, Y. Sato, H. Motoshima, F. Ike, and S. Kaminogawa. 1993. Screening for the immunopotentiating activity of food microorganisms and enhancement of the immune response by *Bifidobacterium adolescentis* M101-4. *Biosci. Biotech. Biochem.* **57:** 2127–2132.
18. Miettinen, M., J. Vuopio-Varkila, and K. Varkila. 1996. Production of human necrosis factor alpha, interleukin-6 and interleukin-10 is induced by lactic acid bacteria. *Infect. Immun*. **64:** 5403–5405.
19. Mitsuoka, T. 1982. Recent trends in research on intestinal flora. *Bifidobact. Microfl*. **1:** 3–24.
20. Namioka, S. 1985. Immunoresponsiveness of newborn piglets and peptidoglycan derived from *Bifidobacterium*. *Bifodobact. Microfl*. **4:** 3–14.
21. Park, M. S., H. W. Moon, and G. E. Ji. 2003. Molecular Characterization of plasmid from *Bifidobacterium longum*. *J. Microbiol. Biotechnol*. **13:** 457–461.
22. Park, S. Y., G. E. Ji, Y. T. Ko, H. K. Jung, Z. Ustunol, and J. J. Pestka. 1999. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. *Int. J. Food Microbiol*. **46:** 231–241.
23. Rafter, J. J. 1995. The role of lactic acid bacteria in colon cancer prevention. *Scand. J. Gastroenterol.* **30:** 497–502.
24. Sasaki, T., S. Fukami, and S. Namioka. 1994. Enhanced resistance of mice to *Escherichia coli* infection induced by administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *J. Vet. Med. Sci*. **53:** 433–437.
25. Sekine, K., E. Watanabe-Sekine, J. Ohta, T. Toida, T. Tatsuki, T. Kawashima, and Y. Hashimoto. 1994. Induction and activation of tumoricidal cells *in vivo* and *in vitro* by the bacterial cell wall of *Bifidobacterium infantis*. *Bifidobact. Microfl*. **13:** 65–77.
26. Sekine, K., J. Ohta, M. Onishi, T. Tatsuki, Y. Shimokawa, T. Toida, T. Kawashima, and Y. Hashimoto. 1995. Analysis of antitumor properties of effector cells stimulated with a cell wall preparation (WPG) of *Bifidobacterium infantis*. *Biol. Pharm. Bull*. **18:** 148–153.
27. Strober, W. 1991. Trypan blue exclusion test for cell viability, pp. A.3.3–4. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Marguiles, E. M. Shevach, and W. Strober (eds.), Current Protocols in Immunology, Greene Pub. and Wiley-Interscience, New York., U.S.A
28. Yasui, H. and M. Ohwaki. 1991. Enhancement of immune response in Peyer's patch cells cultured with *Bifidobacterium breve*. *J. Dairy Sci.* **74:** 1187–1195.
29. Yu, K. W., K. S. Shin, Y. M. Choi, and H. J. Suh. 2004. Macrophage stimulating activity of exo-biopolymer from submerged culture of *Lentinus edodes* with rice bran. *J. Microbiol. Biotechnol.* **14:** 658–664.

J. Microbiol. Biotechnol. (2005), **15**(2), 265–268

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of Bifidobacteria on Production of Allergy-Related Cytokines from Mouse Spleen Cells

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Received: April 13, 2004
Accepted: May 17, 2004

Abstract To study the effect of bifidobacteria on preventing allergy response, levels of IFN-γ, IgG2a, IL-4, and IgG1 were investigated in splenocytes isolated from ovalbumin (OVA)-sensitized allergic mice and BGN4-administered allergy-suppressed mice in the presence of various bifidobacterial strains. Most of the bifidobacteria, except 2A, increased production of Th1-associated immune markers, IFN-γ and IgG2a. In addition, most of the bifidobacteria, except 2A and 19A, decreased production of IL-4, whereas the differences in the production of IgG1 were less pronounced. These results suggest that some strains of bifidobacteria may have the potential to prevent the occurrence of allergy by switching Th1/Th2-type antibodies and/or related cytokines.

Key words: Allergy, *Bifidobacterium*, interferon-γ, interleukin-4, immunoglobulin IgG1, immunoglobulin IgG2a

Allergy, in the form of atopic diseases such as atopic eczema, allergic rhinitis, and asthma, has been increasing in well-developed countries [5]. The Centers for Disease Control and Prevention (CDC) of the U.S.A. estimated 16 million (7.5%) U.S. adults with asthma [3]. The International Study of Asthma and Allergies in Childhood investigated 11,607 Finnish children aged 13–14 years; 10–20% of the children had symptoms of asthma, 15–23% had allergic rhinitis, and 15–19% had atopic eczema [13, 14]. In the Republic of Korea, the prevalence of the symptoms of asthma, rhiniconjunctivitis, and flexural eczema were 8.7%, 10.5%, 7.3% in 6–12-yr-olds, and 8.2%, 10.0%, 3.9% in 12–15-yr-olds, respectively [9].

Differentiation of T-helper (CD4+) cells into two subsets, Th1 and Th2, each with a characteristic profile of cytokine production, is central to the understanding of the pathogenic mechanisms of allergy. Th1 cells produce IFN-γ, IL-2, and tumor necrosis factor-β, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10. The balance of the two types of cells is considered to be important to maintain homeostasis of the host immune system. Once this balance becomes disturbed, various immunological diseases, such as allergies and infections, can occur due to the evasion of host defense mechanisms. Recently, some probiotic strains were reported to alter the balance of immune cell types and their cytokines. Shida *et al.* [15]. showed that *Lactobacillus casei* induced IFN-γ, but inhibited IL-4 and IL-5 secretion, and markedly suppressed total and antigen-specific IgE secretion by ovalbumin (OVA)-stimulated splenocytes. The production of Th1 cell-associated cytokines, IFN-γ and IL-2, by the spleen cells was higher than that by the spleen cells from the control group in the mice fed *L. casei* strain Shirota [12]. These results suggest that some strains of probiotic bacteria may be able to switch the balance of T-helper cells from Th2 to Th1.

Fang *et al.* [4] showed that there were differences in *Bifidobacterium* strains isolated from allergic and healthy infants. Allergic infants were found to have an adult type *Bifidobacerium* flora with a high level of *B. adolecentis*, while the healthy infants had a typical infant *Bifidobacterium* flora with high levels of *B. bifidum* and *B. infantis*. Furthermore, infants with food allergies have been reported to have a disturbed balance between beneficial and potentially harmful bacteria in the large intestine [1, 8], and the development of an aberrant microbial composition in the gut, such as inadequate bifidobacterial biota, is reported to deprive the developing immune system from counter-regulatory signals against Th2-mediated allergic responses [6]. In this context, supplementation of *Bifidobacterium* strains to human hosts may bear some relevance to preventing allergic reactions. The present study was conducted to

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investigate the effect of various *Bifidobacterium* strains on the patterns of T-helper cell-associated antibodies and cytokines using mice spleen cells *in vitro*.

MATERIALS AND METHODS

Mice

Three-week-old C3H/HeJ female mice, weighing 11–13 g, were purchased from Japan SLC (Hamamatsu, Japan) and maintained on ovalbumin-free chow conditions. The mice were kept in plastic cages and allowed free access to water. The temperature and humidity were controlled at 23±1°C and 55±10 %, respectively, and the animals were maintained on a 12:12 h light:dark cycle in an animal environmental control chamber. The animal experimentation guidelines of Seoul National University were followed.

Microorganisms

Bifidobacteria strains were provided by Maeil Co Ltd., and were cultured anaerobically in MRS media (Difco, Detroit, MI, U.S.A.) containing 0.05% L-cysteine (Sigma, St. Louis, MO, U.S.A.) at 37°C for 24 h [7]. For the preparation of the mice diet, all the bacterial cells were collected by centrifugation (Hanil, Seoul, Korea) at 4,000 ×*g* for 40 min at 4°C, and washed twice with sterile phosphate buffer saline. Then, the pellets were lyophilized by freeze-drier (Ilshin, Seoul, Korea), and lyophilized powders were suspended in RPMI medium and boiled at 100°C for 30 min to prepare cell cultures.

Intragastric Antigen Sensitization and Treatment

The mice were deprived of diet for 2 h. Sensitization was performed by intragastric (ig) administration of 50 μg of ovalbumin (OVA) (Sigma, St. Louis, MO, U.S.A.) with 10 μg of cholera toxin (CT) on days 0, 1, 2, 7, and 21 by means of a blunt stainless steel feeding needle. OVA was used as the antigen. The cholera toxin was purchased from Sigma (St. Louis, MO, U.S.A.). The mice were fed with 0.2% of lyophilized *Bifidobacterium* strains in the diet pellets. They were fed experimental bacteria powder 2 weeks before initial sensitization until sacrifice.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokines and Chemokines

Cytokine sandwich ELISA was used to quantify the concentration of soluble cytokine and chemokine proteins according to the manufacturer's instruction. Antibodies for ELISAs were purchased from PharMingen (San Diego, CA, U.S.A.).

Preparation of Cells from Spleen

Splenocytes were isolated from each group of mice sacrificed at week 7. Cells from the spleen were prepared by gently teasing with glass slides and passed through a 200-gauge stainless steel mesh. Red blood cells in the spleen were lysed by 0.83% NH_4Cl-Tris buffer (pH 7.6). Cells were cultured in RPMI 1640 culture media (Gibco BRL, N.Y., U.S.A.) containing 10% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL). The cells were stimulated with ConA (10 μg/ml, Sigma), lyophilized bifidobacteria powder (100 μg/ml) for 48 h in 24-well flat-bottom plates at a density of 5×10^6 cells/ml in RPMI 1640 culture media (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL) and 1% penicillin streptomycin (Gibco BRL) under an atmosphere of 5% CO_2. The supernatant collected was used to measure cytokine production. All cultures were incubated at 37°C in humidified atmosphere with 5% CO_2 (Sanyo, Japan).

RESULTS AND DISCUSSION

In the present study to investigate the immunomodulatory effect of various bifidobacteria on the occurrence of allergy, we modified the peanut allergy murine model by Lee *et al.* [10] and instead employed OVA as a food allergen. Because this OVA-induced allergy murine model was sensitized by only oral challenge, but not by injection, the antigen will permeate only through the intestinal membrane. Therefore, the response of the OVA-induced allergy murine model was expected to be very close to food allergy response. Additionally, the immunomodulatory effect of bifidobacteria was also investigated in the spleen cells from BGN4-treated mice, in which the occurrence of OVA-induced allergy was suppressed by the simultaneous administration of probiotic strain *Bifidobacterium* sp. BGN4. The levels of IFN-γ, IL-4, IgG1, and IgG2a in the splenocyte culture supernatant were measured as immune markers for the study.

In mice, IL-4 is the most important signature cytokine of Th2 cells. It induces a class switch in B-cells to the antibody classes IgE and IgG1, while IFN-γ from Th1 cells stimulate the production of IgG2a antibodies [2]. Therefore, IgG2a is considered to be a Th1-associated antibody, while IgG1 is considered to be a Th2-associated antibody. The IgG1/IgG2a ratio has frequently been used to detect switching of Th1/Th2 balance. Indeed, it was reported that allergic mice possessed a high serum ratio of IgG1/IgG2a [9].

The production of IFN-γ and IgG2a in the experimental groups was higher than that of the control group, with the exception of 2A. Specifically, 6A, 7A, and 8A showed marked increases of IFN-γ levels in the spleen cells from both OVA-induced mice and BGN4-administered mice. In comparison, 2A showed the highest level of IgG2a in the splenocytes and highly stimulated the production of IL-4 and IgG1 in the splenocytes isolated from OVA-induced mice and BGN4-administered mice. The 19A highly stimulated

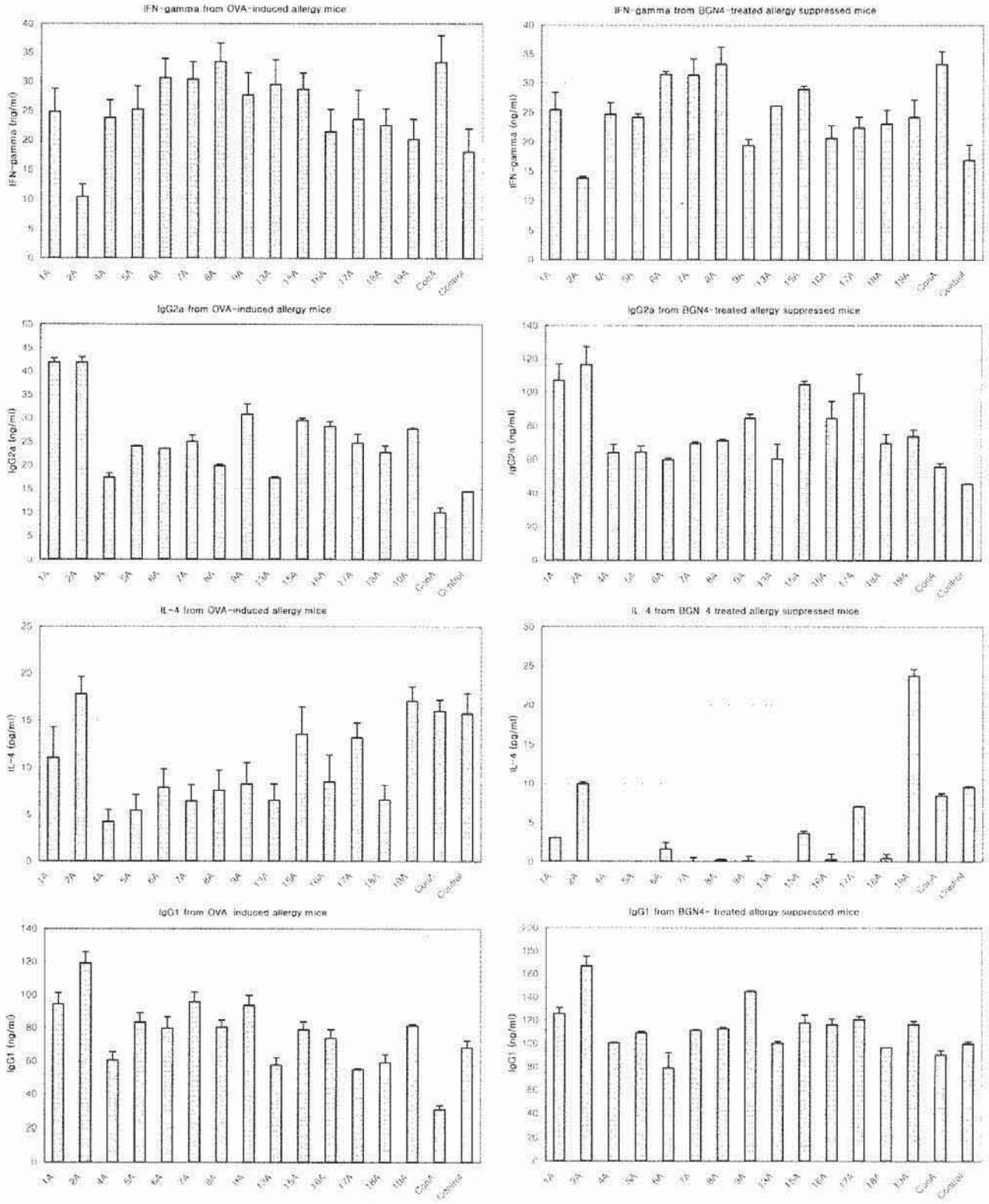

Fig. 1. The production of IFN-γ, IgG2A, IL-4, and IgG1 by spleen cells *in vitro*.
C3H/HeJ mice were orally sensitized on days 0, 1, 2, 7, 14, and 21 with 50 μg of ovalbumin and 10 μg of choleratoxin at a total volume of 0.2 ml. The mice were then fed a diet containing (wt/wt) 0.2% *Bifidobacterium bifidum* BGN4 for 21 days. Allergy mice were fed a diet containing (wt/wt) 0.2% cornstarch powder instead of bacteria powder. Spleen cells were collected on day 30 and were cocultured with various *Bifidobacterium* strains powder for 72 h. The amount of each of IFN-γ, IgG2A, IL-4, and IgG1 in the supernatant was measured by ELISAs. The data are representative of one of two independent experiments. Bars represent mean±SEM of triplicate cultures.

the production of IL-4 in the splenocytes from OVA-induced mice and BGN4-administered mice, but did not show any marked increase in the levels of IgG1, compared with other bifidobacteria.

Overall, most of the strains, except 2A and 19A, suppressed the production of IL-4, when compared to that of the control group. However, the differences in the production of IgG1 were less pronounced between the experimental groups.

The ratio of IgG1/IgG2a rather than individual levels of IgG1 and IgG2a is considered to be important to compare immunomodulatory effect on the occurrence of allergy and hypersensitivity. In correlation to the findings of Marinaro *et al.* [11], the results from the present study suggest that the reversal of IgG1/IgG2a ratio was due to markedly increased IgG2a synthesis rather than to the reduction of IgG1. This phenomenon was observed in both OVA-induced allergy mice and BGN4-treated allergy-suppressed mice. We also demonstrated that the degree of augmentation of IFN-γ and the reduction of IL-4 was dependent on the strain, in support of Fang *et al.* [4] who reported differences in *Bifidobacterium* strains isolated from allergic and healthy infants. The bifidobacteria strains in the present study that showed strong increases of Th1-type response might be possible candidates to be used as probiotics for allergy prevention. However, further experiments should be conducted to elucidate the detailed mechanisms by which *Bifidobacterium* inhibits allergy response in experimental animals and clinical human studies.

Acknowledgment

This work was supported by the Korean Ministry of Science and Technology (MI-0303-00-0098).

References

1. Bjorksted, B., P. Naaber, E. Sepp, and M. Mikelsaar. 1999. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin. Exp. Allergy* **29:** 342-346.
2. Centers for Disease Control and Prevention. 2002. *Asthma Prevalence and Control Characteristics by Race/Ethnicity* - United States.
3. Coffman, R. L., D. A. Lebmann, and P. Rothman. 1993. Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* **54:** 229-270
4. Fang, H., A. C. Ouwehand, E. Isolauri, H. Morita, M. Hosoda, H. Hashimoto, T. Fuse, K. Mizumachi, J. I. Kurisaki, Y. Benno, and S. Salminen. 2001. Bifidobacteria isolated from allergic and healthy infants: Differences in taxonomy, mucus adhesion and immunomodulatory effects. *International Conference of Intestinal Bacteriology. B-04.*
5. Holgate, S. T. 2004. The epidemic of allergy and asthma. *J. R. Soc. Med.* **97:** 103-110.
6. Kalliomäki, M., P. Kirjavainen, E. Eerola, P. Kero, S. Salminen, and E. Isolauri. 2001. Distinct patterns of neonatal gut microflora in infants developing atopy. *J. Allergy Clin. Immunol.* **107:** 129-134.
7. Kim, J. H., M. S. Park, and G. E. Ji. 2003. Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 cells. *J. Microbiol. Biotechnol.* **13:** 276-281.
8. Kirjavainen, P. V., T. Arvola, S. J. Salminen, and E. Isolauri. 2002. Aberrant composition of gut microbiota of allergic infants: A target of bifidobacterial therapy at weaning. *Gut* **51:** 51-55.
9. Lee, S. I., M. H. Shin, H. B. Lee, J. S. Lee, B. K. Son, Y. Y. Koh, K. E. Kim, and Y. O. Ahn. 2001. Prevalence of symptoms of asthma and other allergic diseases in Korean children: A nationwide questionnaire survey. *J. Korean Med. Sci.* **16:** 155-164.
10. Lee, S. Y., C. H. Huang, T. F. Zhang, B. H. Schofield, A. W. Burks, G. A. Bannon, H. A. Sampson, and X. M. Li. 2001. Oral administration of IL-12 suppresses anaphylactic reactions in a murine model of peanut hypersensitivity. *Clin. Immunol.* **101:** 220-228.
11. Marinaro, M., P. N. Boyaka, F. D. Finkelman, H. Kiyono, R. J. Jackson, E. Jirillo, and J. R. McGhee. 1997. Oral but not parenteral interleukin (IL)-12 redirects T helper 2 (Th2)-type responses to an oral vaccine without altering mucosal IgA responses. *J. Exp. Med.* **185:** 415-426.
12. Matsuzaki, T., R. Yamazaki, S. Hashimoto, and T. Yokokura. 1998. The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. *J. Dairy Sci.* **81:** 48-53.
13. Pekkanen, J., S. T. Remes, T. Husman, M. Lindberg, M. Kajosaari, A. Koivikko, and L. Soininen. 1997. Prevalence of asthma symptoms in video and written questionnaires among children in four regions of Finland. *Eur. Respir. J.* **10:** 1787-1794.
14. Remes, S. T., M. Korppi, M. Kajosaari, A. Koivikko, L. Soininen, and J. Pekkanen. 1998. Prevalence of allergic rhinitis and atopic dermatitis among children in four regions of Finland. *Allergy* **53:** 682-689.
15. Shida, K., K. Makino, A. Morishita, K. Takamizawa, S. Hachimura, A. Ametani, T. Sato, Y. Kumagai, S. Habu, and S. Kaminogawa. 1998 *Lactobacillus casei* inhibits antigen induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. *Int. Arch. Allergy Immunol.* **115:** 278-287.

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FEMS Immunology and Medical Microbiology xxx (2005) xxx–xxx

FEMS Immunology and Medical Microbiology

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Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model

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Received 12 December 2004; received in revised form 6 April 2005; accepted 2 May 2005

First published online

Abstract

This study investigated whether orally administered probiotic bacteria (*Bifidobacterium bifidum* and *Lactobacillus casei*) and a gram-negative bacterium (*Escherichia coli*) function as allergic immune modulators to prevent food allergy, according to the hygiene hypothesis. C3H/HeJ mice were sensitized with ovalbumin (OVA) and cholera toxin for 5 weeks. After sensitization, the OVA-induced mice that were not treated with bacteria had significantly increased levels of OVA-specific IgE, total IgE, and IgG1 in sera, as well as scab-covered tails. In comparison, groups treated with *B. bifidum* BGN4 (BGN4), *L. casei* 911 (*L. casei*), or *Escherichia coli* MC4100 (*E. coli*) had decreased levels of OVA-specific IgE, total IgE, and IgG1, and decreased levels of mast cell degranulation and tail scabs. OVA-specific IgA levels were decreased in BGN4- and *L. casei*-treated groups. In conclusion, administration of *E. coli*, BGN4, or *L. casei* decreased the OVA-induced allergy response. However, a normal increase in body weight was inhibited in the *E. coli*-treated mice and in the montreated mice groups during allergy sensitization. Thus, BGN4 and *L. casei* appear to be useful probiotic bacteria for the prevention of allergy.

Keywords: Allergy; *Bifidobacterium*; *Escherichia coli*; *Lactobacillus casei*; Immunoglobulin E

1. Introduction

The prevalence of atopic disease has increased rapidly around the world during recent decades [1,2]. Food allergy is not entirely separate from other allergic reactions such as eczema, asthma, and rhinoconjunctivitis. An incidence of 90.5% of food allergy in patients with atopic dermatitis and an increased risk of clinical sensitivity to food in asthmatic patients were reported [3,4].

The so-called hygiene hypothesis attributes the increasing prevalence of atopic diseases [5,6] to disease reduction resulting from vaccinations and improved hygiene in industrialized countries. Specifically, the hygiene hypothesis suggests that modern methods of hygiene and sanitation have decreased children's exposure to certain microbes and negative bacteria. The neonatal and early childhood periods are believed to be critical periods for the establishment of the Th1–Th2 balance. Early infections during these stages are believed to result in a Th1-biased immunity and to prevent the induction of the pro-allergic Th2 system [7]. The intestinal microflora established during infancy may be a source for the induction of immune deviation, and the flora composition may determine whether allergy disorders develop [8]. Children who developed allergy during

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0928-8244/$22.00 © 2005 Published by Elsevier B.V. on behalf of the Federation of European Microbiological Societies.
doi:10.1016/j.femsim.2005.05.005

the first two years of life were less often colonized with enterococci, bifidobacteria, and bacteroides, and had higher stool counts of *Staphylococcus aureus* and *Clostridium* in comparison with healthy infants [9].

In an attempt to reverse these possible effects of reduced microbial exposure in early life, probiotics have been administered to infants. Probiotics are traditionally defined as live microbial food supplements that improve intestinal microbial balance [10]. The frequency of atopic eczema in a *Lactobacillus* GG-treated group was half that in a placebo-treated group, suggesting that *Lactobacillus* GG effectively prevented early atopic disease in children at high risk [7]. Another study showed that *Lactobacillus casei* might inhibit antigen-induced IgE production by inducing interleukin-12 (IL-12) secretion by macrophages [11]. Specific microbes in the gastrointestinal of the host may promote potentially anti-allergenic processes through induction of Th1 type immunity [12,13] and/or enhance the production of transforming growth factor-β [14,15], which plays an essential role in the suppression of T-helper 2 cell-induced allergy [10,16]. The splenic production of Th1 cell-associated cytokines, such as interferon-γ and interleukin-2, was higher in mice fed the *L. casei* Shirota strain than in the control group [11]. Natural killer (NK) cells [17] and cytotoxic T-cells [18] were also stimulated by *L. casei*. These results suggest that *L. casei* enhances cellular immunity, in which Th1 cells may play an important role. However, the precise mechanisms by which probiotics inhibit allergy remain unknown.

To compare the utility of probiotic and non-probiotic bacteria for allergy prevention, we investigated the effects of the *Bifidobacterium* strain *Bifidobacterium bifidum* BGN4, the *Lactobacillus* strain *L. casei* 911, and the *Escherichia coli* strain *E. coli* MC4100 in an ovalbumin (OVA)-induced allergy mouse model. Additionally, we investigated mechanisms relating to the immunological effect of suppression on the OVA-induced allergy response. To our knowledge, this is the first report to assess the effect of probiotics in an orally sensitized food allergy animal model. Thus, the method described in the present study is probably better adapted to studying the effect of probiotics on food allergies than previous experimental studies in which antigens were intraperitoneally injected.

2. Materials and methods

2.1. Mice

Three-week-old female C3H/HeJ mice weighing 11–13 g were purchased from Japan SLC (Hamamatsu, Japan) and maintained on OVA-free chow. Mice were sensitized at 5 weeks of age and each group included six mice. Mice were kept in plastic cages, allowed free access to water, and maintained on a 12:12 h light:dark cycle in an environmentally controlled animal chamber. The temperature and humidity were controlled at 23 ± 1 °C and 55 ± 10%, respectively. The animal experimentation guidelines of Seoul National University were followed.

2.2. Microorganisms

B. bifidum BGN4 and *L. casei* 911 were used as they were previously suggested to be promising probiotic strains with regard to anti-cancerogenic activity [19,20] and to have the ability to attach to human epithelial cell lines [21]. *E. coli* MC4100 was used as gram-negative bacterium. *Bifidobacterium* and *Lactobacillus* were anaerobically cultured in Lactobacilli-MRS broth (Difco, Detroit, MI, USA) containing 0.05% L-cysteine (Sigma, St. Louis, MO, USA) at 37 °C for 24 h. The *E. coli* MC4100 strain was cultured aerobically in LB broth (Criterion, CA, USA) at 37 °C for 24 h. To prepare the mouse diets, bacterial cells were collected by Mega 21R centrifuge (Hanil, Seoul, Korea) at 4000*g* for 40 min at 4 °C, and washed twice with sterile phosphate buffer saline. Then the pellets were dried by the FD5508 lyophilizer (Ilshin, Seoul, Korea) and mixed with the mouse diet.

2.3. Intragastric antigen sensitization and treatment

Mice were deprived of diet for 2 h preceding the oral sensitization. Sensitization was performed by intragastric (ig) administration of 50 μg OVA with 10 μg of cholera toxin (CT) on days 0, 1, 2, 7, 21, and 35 using a stainless steel blunt feeding needle. OVA (Sigma, St. Louis, MO, USA) was used as the antigen. CT and concanavalin A (Con A) were purchased from Sigma (St. Louis, MO, USA). Five groups of mice were used in this study (Fig. 1). Mice in groups 2–5 were gavaged with 0.2 ml phosphate-buffer saline solution (PBS, pH 7.2) containing OVA and CT. Mice in the naïve group (group 1) were gavaged with PBS without OVA and CT as a negative control. Mice in groups 2–5 were subjected to the same OVA sensitization. Then mice in groups 3–5 were administered bacterial powder. Mice in group 2 received OVA and CT but no bacteria as a sham control. Bacteria-treated mice were fed 0.2% of lyophilized *B. bifidum* BGN4 (BGN4), *L. casei* 911, or *E. coli* MC4100 via a diet pellet. The concentration of bacteria was tested in a preliminary study, which was not published. Mice were fed the experimental bacterial powders for 7 weeks, starting 2 weeks before the initial sensitization, until they were finally sacrificed. To determine serum antibody responses, tail vein blood was collected weekly after the initial sensitization. Sera were stored at −80 °C.

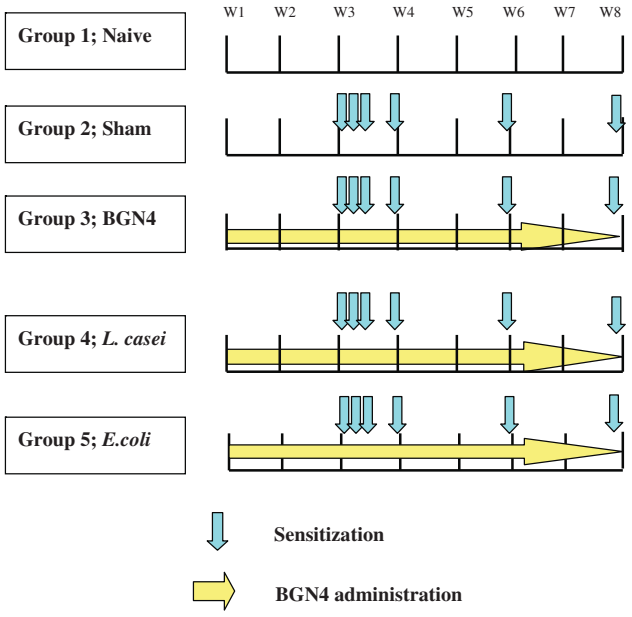

Fig. 1. Experimental protocol: intragastric ovalbumin sensitization and bacteria administration. Mice were sensitized on weeks 3, 4, 6, and 8 with ovalbumin and choleratoxin. Mice in group 3, group 4, and group 5 were fed 0.2% lyophillzed BGN-4, *L. casei*, or *E. coli* in diet pellet for 8 weeks starting 2 weeks before initial sensitization until sacrifice, respectively. The naïve mice in group 1 served as a negative control. Mice in group 2 received PBS buffer instead of ovalbumin and choleratoxin as a sham treatment.

2.4. Measurement of serum OVA-specific IgE, IgG1, IgG2a, total IgE, IgG1, IgG2a, spleen IL-5, IL-13, IgG1, and IgG2a levels

Tail vein blood was obtained weekly following initial sensitization. Sera were collected and stored at −80 °C. Levels of OVA-specific-IgE, IgG1, and IgG2a were measured using an enzyme-linked immunosorbent assay (ELISA). Briefly, Nunc-Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 5 μg ml^{-1} of OVA in coating buffer, pH 9.6 (Sigma, St. Louis, MO, USA) overnight at 4 °C. Plates were blocked and washed. Samples were added to the plates and incubated overnight at 4 °C. Plates were washed, and biotinylated rat anti-mouse IgE, IgG1, or IgG2a monoclonal antibodies (2 μg ml^{-1}) were added to the plates for detection of OVA-specific IgE, IgG1, and IgG2a, respectively, for 1 h at room temperature. The reactions were developed with the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Fluka, Neu-Ulm, Switzerland) for 30 min at room temperature. The color reactions were stopped with 6 N H_2SO_4 and read at 450 nm. Equivalent levels of IgE, IgG1, or IgG2a were calculated by comparison with a reference curve generated with standards of total mouse IgE, IgG1, or IgG2a, respectively.

Total IgE, total IgG1, and total IgG2a from serum were determined using ELISAs. To detect these antibodies, plates were coated with 2 μg ml^{-1} of rat monoclonal anti-mouse IgE, IgG1, or IgG2a, respectively. Serial dilutions of serum were added and then followed by addition of 100 μl of a biotinylated rat monoclonal anti-mouse IgE, IgG1, or IgG2a. The houseradish peroxidase (HRP)-conjugated streptavidin, described above, was used for detection, and plates were developed with the TMB substrate. IL-5 and IL-13 levels from spleen culture supernatants were detected using ELISA as described above. All of the antibodies used in this study were purchased from Pharmingen (San Diego, CA, USA).

2.5. Measurement of OVA-specific and total fecal IgA

Extracts of fecal pellets were prepared as described by Marinaro et al. [22]. In brief, 100 mg of pellet was mixed with 1 ml of PBS containing 0.1% NaN_3 and incubated at 4 °C for 2 h. Then the pellet was vortexed for 10 min. After centrifugation (4000*g*, 20 min), supernatants were collected and stored at −70 °C. For the assays, plates were coated with 5 μg ml^{-1} of OVA in coating buffer. After washing and blocking, 100 μl of fecal extracts were added to individual wells and incubated overnight at 4 °C. Plates were then washed, and biotinylated rat anti-mouse IgA monoclonal antibodies (2 μg ml^{-1}) were added to the plates and incubated for an additional hour at room temperature. After washing, avidin-peroxidase was added for 1 h at room temperature. The reactions were developed with TMB (Fluka, Neu-Ulm, Switzerland) for 30 min at room temperature. The color reactions were stopped with 6 N H_2SO_4 and read at 450 nm. Equivalent levels of IgA were calculated by comparison with a reference curve generated with a mouse total IgA standard.

For measurement of total IgA, plates were coated with rat anti-mouse IgA capture antibodies (2 μg ml^{-1}) in coating buffer. Plates were then blocked and washed in the manner described above. Fecal extracts (1:50 dilutions) were added to the plates and incubated overnight at 4 °C. Plates were washed and then 100 μl of biotinylated rat anti-mouse IgA were added to each well. Subsequent steps were as described above. IgA levels were calculated from a reference curve generated with a mouse total IgA standard.

2.6. Histology

Mast cell degranulation during food allergy response was assessed by examination of ear and tongue samples collected immediately after sacrifice. Tissues were fixed in 10% neutral buffered formalin, and paraffin sections were stained with toluidine blue (Sigma, St. Louis, MO, USA). Histologic scores were counted in a double-blind manner; observers unaware of sample identities counted the degranulated mast cells in sections

from mouse ears and tongues using light microscopy (100×). Degranulated mast cells were defined as toluidine blue positive cells with five or more distinctly stained granules completely outside of the cells. Each ear sample contained 200–900 mast cells.

2.7. Assessment of hypersensitivity reactions

Allergic symptoms were evaluated after sacrifice utilizing a scoring system: 0, no symptoms; 1, puffiness of the tail; 2, 1–2 scabs on the tail; 3, 3–4 scabs on the tail; 4, 5–6 scabs on the tail; 5, more than 7 scabs on the tail. Scoring of symptoms was performed in a blind manner; scores were evaluated by 10 individuals unaware of sample identities.

2.8. Statistical analysis

All data are presented as the means ± standard error of mean (SEM), indicated by bars in the figures. Data were analyzed using SAS (Release 8.01, O, USA). Differences between immunoglobulin and cytokine levels in the groups were analyzed by ANOVA followed by Duncan's multiple range test for multiple comparisons. p values <0.05 were considered significant.

3. Results

3.1. Effect of BGN4, L. casei, and E. coli on IgE production

To monitor the effects of the three bacterial treatments, sera were obtained from each group of mice every week following OVA sensitization. The OVA-specific IgE levels in sera from each group at week 7, measured by ELISA, are presented in Fig. 2B. All three groups administered 0.2% of BGN4, *L. casei*, or *E. coli* had significantly lower OVA-specific IgE levels than the sham group.

Total serum IgE concentrations were dramatically increased in the sham group at weeks 6 and 7 (Fig. 2A). However, total IgE levels in all three bacterial-treated groups were not increased, and were significantly lower than the sham group at weeks 6 and 7. The total serum IgE levels in the three treated groups were not significantly different at week 7 from the levels in naïve mice (naïve, 295 ± 25 ng ml^{-1}; BGN4, 389 ± 31 ng ml^{-1}; *L. casei*, 333 ± 69 ng ml^{-1}; *E. coli*, 314 ± 78 ng ml^{-1}).

3.2. Allergic symptoms in the tail

Mice sensitized with OVA and CT had numerous tail injuries. After administration of OVA and CT, sham-treated mice started to scratch their tails, resulting in severe injuries and bleeding (Fig. 3A). The severity of OVA-induced allergic reactions in mice was scored (Fig. 3B). The severity of tail injuries was significantly reduced in the BGN4-, *L. casei*-, and *E. coli*-treated groups compared with the sham group. BGN4 and *L. casei* administration, but not *E. coli* administration, reduced the severity of symptoms in ear tissues.

3.3. Mast cell degranulation

Histologic analysis of ear and tongue tissues revealed a significant increase in the number of degranulated mast cells in OVA-immunized mice compared with naïve

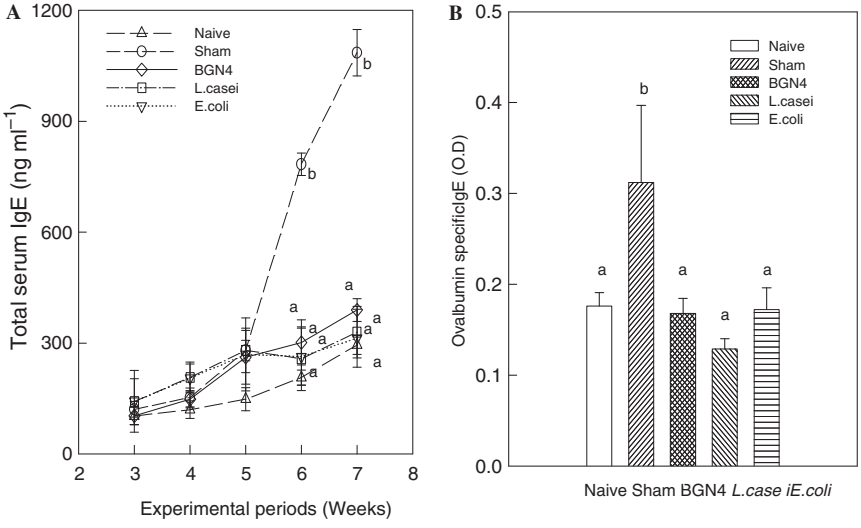

Fig. 2. Effect of bacteria administration on production of total IgE and ovalbumin-specific IgE in serum from ovalbumin-sensitized mice. Sera from all groups of mice were obtained weekly following initial ovalbumin sensitization. IgE levels were determined by ELISAs. Ovalbumin-specific IgE levels were determined at week 7. Data are shown as means ± SEM of six mice per group. Different letters indicate significant differences determined by Duncan's multiple range test ($p < 0.05$).

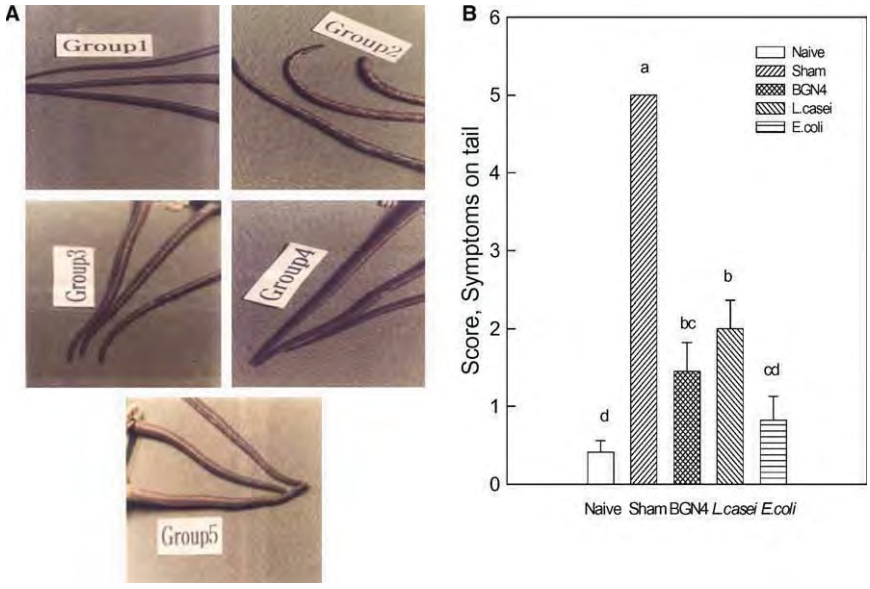

Fig. 3. Severity of allergy symptoms on the tail from ovalbumin-sensitized mice treated with BGN4, *L. casei*, or *E. coli*. Immunized mice showed marked tail bruising and scabs. (A) Pictures of the tail from ovalbumin-sensitized mice treated with bacteria. Group 1, naïve; Group 2, sham; Group 3, BGN4-treated; Group 4, *L. casei*-treated; Group 5, *E. coli*-treated. (B) Severity of allergy symptoms on tail of ovalbumin-sensitized mice was evaluated utilizing a scoring system, and scoring was performed in a blind manner ($p < 0.05$).

mice (Fig. 4). Consistent with the elevated OVA-specific IgE levels in sham-treated mice, the percentage of degranulated mast cells in the sham group was much greater than the percentage in the naïve and bacteria-treated groups. In the ear tissues, BGN4 or *L. casei* administration, but not *E. coli*-administration, reduced the severity of symptoms.

3.4. OVA-specific mucosal IgA

OVA-specific IgA levels in sham-treated mice were more than threefold higher than in BGN4- or *L. casei*-treated animals (Fig. 5). OVA-specific IgA in *E. coli*-treated animals was higher than in BGN4- or *L. casei*-treated animals (naïve, 101 ± 15 μg ml^{-1}; sham,

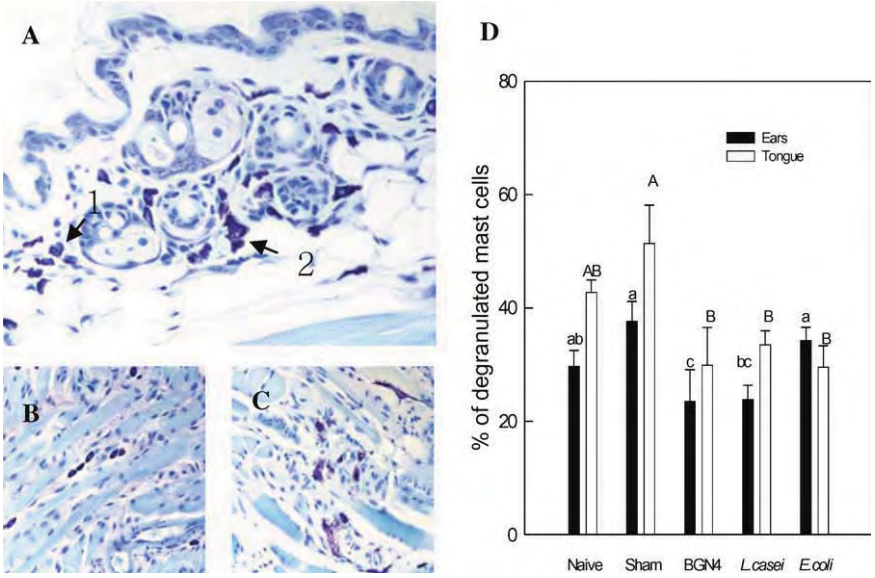

Fig. 4. Effect of bacteria on percentage of mast cell degranulation in ovalbumin-sensitized mouse model. (A) Non-degranulated (arrow 1) and degranulated mast cells (arrow 2) in ear sample. (B) Non-degranulated mast cells in tongue sample. (C) Degranulated mast cells in tongue sample from ovalbumin-induced mice. (D) Percentage of degranulated mast cells in ear and tongue samples. Two to 900 mast cells infiltrated in the tissues were counted, and mast cells with more than five released granules were defined as degranulated cells. Data shown are means ± SEM of six mice per group. Different letters indicate significant differences ($p < 0.05$, in ear and tongue sample).

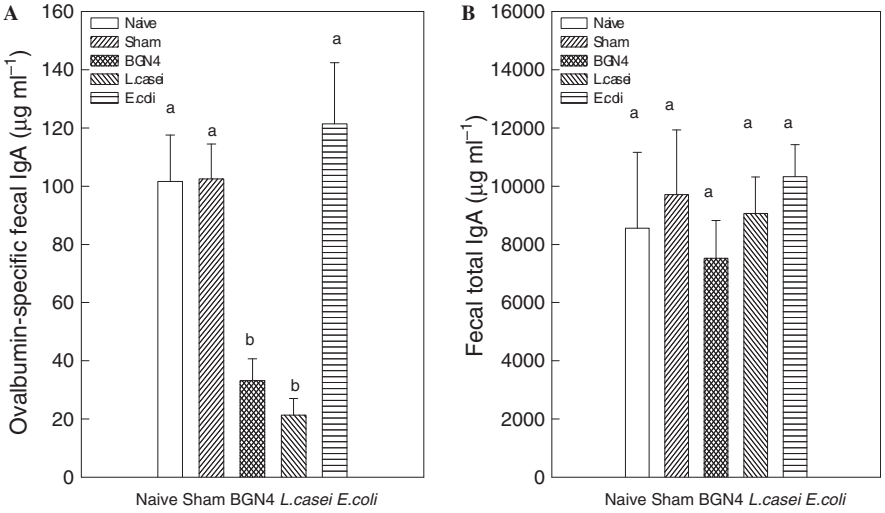

Fig. 5. Effect of bacteria on the production of ovalbumin-specific IgA (A) and total IgA (B) in fecal sample from ovalbumin-sensitized mice and bacteria-treated mice. Fresh fecal pellets from each group were collected. Fecal extracts were prepared and ovalbumin-specific IgA levels were detected by ELISAs. Data shown are means ± SEM of six mice per group. Different letters indicate significant differences ($p < 0.05$).

102 ± 11 μg ml^{-1}; BGN4, 33 ± 7 μg ml^{-1}; *L. casei*, 21 ± 5 μg ml^{-1}; *E. coli*, 121 ± 21 μg ml^{-1}). However, total mucosal IgA levels did not differ significantly among groups.

3.5. Alteration of IgG1 and IgG2a levels in sera

The levels of OVA-specific IgG1 were significantly lower in BGN4- and *L. casei*-treated mice but not in *E. coli*-treated mice compared to sham mice at week 7 (naïve, 1099 ± 495 ng ml^{-1}; sham, $13{,}183 \pm 4223$ ng ml^{-1}; BGN4, 5197 ± 1017 ng ml^{-1}; *L. casei*, 3353 ± 517 ng ml^{-1}; *E. coli*, 9531 ± 1811 ng ml^{-1}). The levels of total IgG1 in the BGN4-, *L. casei*-, and *E. coli*-treated groups were significantly lower than in sham mice (naïve, 70 ± 15 μg ml^{-1}; sham, 139 ± 36 μg ml^{-1}; BGN4, 62 ± 5.8 μg ml^{-1}; *L. casei*, 67 ± 15 μg ml^{-1}; *E. coli*, 55 ± 10 μg ml^{-1}). However, OVA-specific IgG2a and total IgG2a levels were not significantly different between the sham and bacteria-treated groups (Fig. 6).

3.6. Effect of BGN4, L. casei, or E. coli on body weight

The initial mean body weights did not differ significantly among the groups (Table 1). Although the body weight of the sham group was greater than that of the naïve group at week 2, the mean body weight of the sham group decreased below that of the naïve group at week 7. On the other hand, the mean body weights of the groups treated with BGN4 or *L. casei* were similar to the mean weights of the naïve group. The mean body weight of the *E. coli*-treated group was consistently lower than that of the naïve group during the experimental period.

4. Discussion

Our modified allergy model was developed from a previously described peanut allergy murine model [23]. The oral sensitization method using OVA as the allergen was employed, since it most closely mimics the route through which human food allergies develop. The normal immune response to dietary proteins is associated with the induction of oral tolerance, and when this active immune suppression is abrogated, adverse reactions to food proteins may arise. Because oral tolerance is obtained on the mucosal site of the gastrointestinal system, studies on food allergy need to be performed by administrating allergens through the intragastric or oral route. Although the immune responses of mouse are not exactly the same as those of human and the mouse model is limited for the scoring of allergic symptoms, murine models provide a useful tool to identify and test new therapeutic strategies, and to expand our knowledge of mechanisms underlying the development of food allergy [24].

Though the precise pathogenic mechanisms involved in food allergy after ingestion of OVA are unknown, the increased OVA-specific IgE production and elevated mast cell degranulation suggest that allergic response was successfully induced, and that activated mast cells might have contributed, at least in part, to the symptoms of food allergy response.

In the present study, BGN4, *L. casei*, and *E. coli*, when administered orally prior to OVA-sensitization, inhibited total IgE production and markedly reduced OVA-specific IgE levels. Additionally, tongue samples from bacteria-treated mice had significantly decreased numbers of degranulated mast cells compared with the sham group. These results demonstrate that bacteria

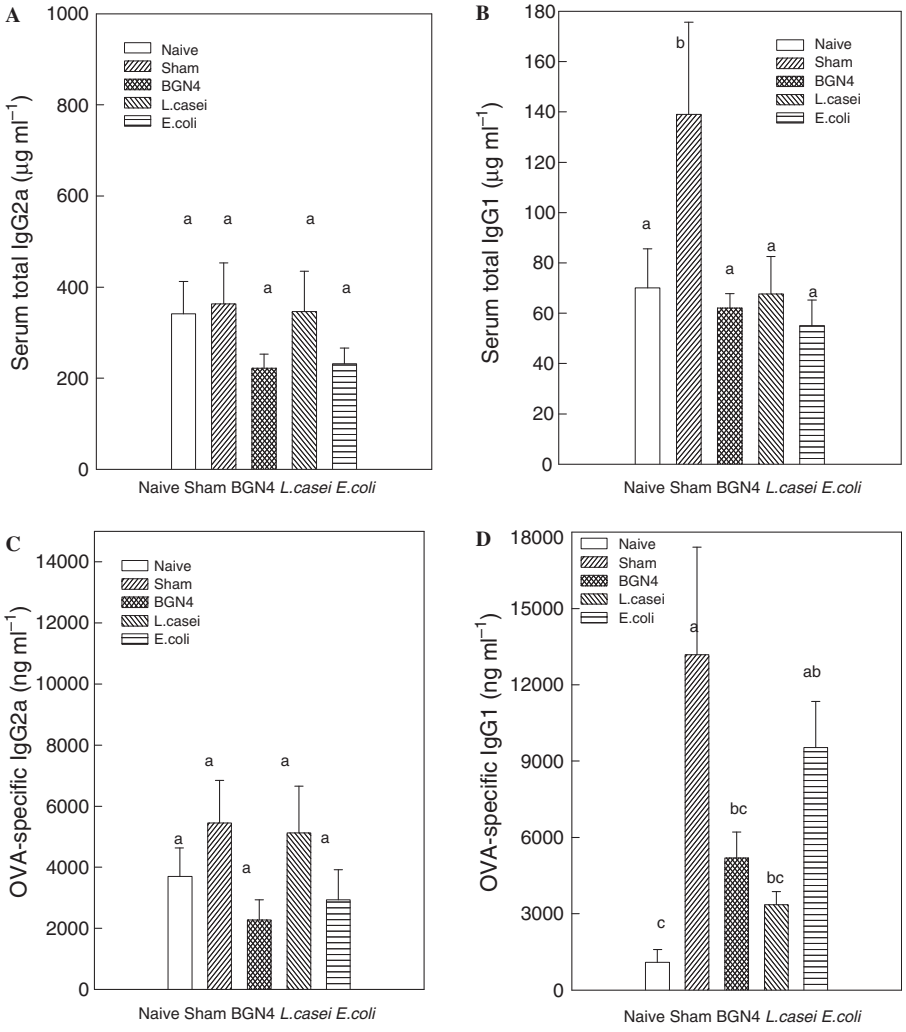

Fig. 6. Effect of bacteria on the production of ovalbumin-specific IgG2a, ovalbumin-specific IgG1, total IgG2a, and total IgG1 in serum from ovalbumin-sensitized mice and bacteria-treated mice. Levels of antibodies were detected by ELISAs. Data shown are means ± SEM of six mice per group. Different letters indicate significant differences ($p < 0.05$).

Table 1
Body weight of mice fed a diet containing (wt/wt) 0.2% of BGN4, *L. casei*, or *E. coli* lyophilized powder per mouse for 7 weeks[A]

Groups	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Naïve	16.4 ± 0.2^{c}	17.8 ± 0.2^{b}	21.6 ± 0.4^{a}	23.1 ± 0.3^{a}	23.3 ± 0.5^{a}	23.8 ± 0.4^{a}
Sham	17.7 ± 0.3^{b}	21.1 ± 0.6^{a}	21.3 ± 0.3^{a}	22.4 ± 0.6^{ab}	21.6 ± 0.4^{ab}	21.6 ± 0.4^{bc}
BGN-4	18.7 ± 0.5^{a}	19.0 ± 0.4^{b}	20.0 ± 0.6^{b}	21.3 ± 0.7^{bc}	22.3 ± 0.9^{a}	22.7 ± 0.9^{ab}
L. casei	18.0 ± 0.3^{ab}	21.0 ± 0.3^{a}	20.5 ± 0.3^{ab}	21.3 ± 0.2^{bc}	21.9 ± 0.6^{ab}	23.2 ± 0.4^{ab}
E. coli	18.0 ± 0.3^{ab}	18.6 ± 0.4^{b}	18.8 ± 0.3^{c}	20.2 ± 0.4^{c}	20.2 ± 0.4^{b}	20.3 ± 1.0^{c}

Values are given as means ± SEM of six mice per group. Different superscripts indicate significant differences ($p < 0.05$).
[A] Experimental diets: naïve, 0.2% cornstarch; sham, 0.2% cornstarch; BGN-4, 0.2% *Bifidobacterium bifidum* BGN-4; *L. casei*, 0.2% *Lactobacillus casei* 911; *E. coli*, 0.2% *Escherichia coli* MC4100.

administration inhibited OVA-specific IgE synthesis and thereby reduced the intensity of allergy symptoms.

Interestingly, tail wounds (clearly induced by oral OVA sensitization in our mouse model) have not been reported in other orally sensitized animal models. Because sera were collected via the tail vein every week, all the mice had tail wounds. Wounds in the naïve group were gradually resolved without treatment, while wounds in the sham group worsened. Just following sensitization with OVA and CT, mice in the sham group, but not the groups treated with BGN4, *L. casei*, or *E. coli*, started scratching their own tails with their teeth, which caused the tail wounds to bleed. Therefore, we concluded that the tail wounds and itching were genuine

OVA-induced allergic responses and that the experimental bacterial treatments prevented these OVA-induced allergic symptoms.

Hessel et al. [25] showed that gram-positive bacteria and gram-negative bacteria produced different cytokines in human peripheral blood cells. The differences in OVA specific fecal IgA levels and OVA specific serum IgG1 levels among the experimental bacteria suggests that these bacteria inhibit allergy responses in different ways.

BGN4 and *L. casei* apparently exerted tight control over Th1 action, and thereby repressed the production of OVA-specific IgE, IgA, and IgG1. Lower levels of IgA in BGN4- and *L. casei*-treated groups might have been partially due to amelioration of CT-induced mucosal stimulation. CT, in combination with IL-4, has been reported to increase the IgG1 response of lipopolysaccharide (LPS)-activated spleen B cells in vitro [26]. Because C3H/HeJ mice are known to be defective in Toll-like-receptor 4 (TLR4) (Toll-like-receptor) and unresponsive to LPS, components other than LPS or mechanisms not directly related to the LPS-TLR4-dependent signaling pathways may play roles in the suppression of IgE and allergy symptoms in the *E. coli*-treated group. Even in experiments that used LPS responsive animal models, the effects of LPS on allergy were not always the same. Park et al. [27] reported that LPS administration prevented development of Th2 responses, pulmonary inflammation, and airway hyperresponsiveness. However, other studies showed that TLR4 might be necessary for the optimal development of Th2 responses rather than Th1 responses [28].

Normal growth in terms of weight was achieved in the mouse groups fed either BGN4 or *L. casei*, but not *E. coli*. Normal absorption of nutrients in the gastro intestinal tract might have been inhibited by the allergy response. The suppression of the allergy response by BGN4 and *L. casei* might have contributed to normal growth, whereas the pathogenic nature of *E. coli* might have inhibited the normal growth of the experimental mice.

In conclusion, the present results demonstrate that oral administration of *Bifidobacterium* or *Lactobacillus* prevents IgE-mediated OVA-hypersensitivity and maintains normal growth of OVA-sensitized mice, and that traditional allergy symptoms were clearly blocked by the bacterial treatments. Additional investigations should help to elucidate the mechanisms involved in OVA-hypersensitivity and identify ways to ameliorate OVA-hypersensitivity.

Acknowledgement

This work was supported by the Korean Ministry of Science and Technology (NRL, MI-0302-00-0098).

References

[1] Von Mutius, E. (1998) The raising trends in asthma and allergic disease. Clin. Exp. Allergy 28, 45–49.
[2] Lee, S.I., Shin, M.H., Lee, H.B., Lee, J.S., Son, B.K., Koh, Y.Y., Kim, K.E. and Ahn, Y.O. (2001) Prevalences of symptoms of Asthma and other allergic diseases in Korean children: a nationwide questionnaire survey. J. Korean Med. Sci. 16, 155–164.
[3] Ogura, Y., Ogura, H. and Zusi, N. (2001) The incidence of food allergy in atopic dermatitis. Arerugi 50, 621–628.
[4] Aba-Alkhail, B.A. and Ei-Gamal, F.M. (2000) Prevalence of food allergy in asthmatic patients. Saudi Med. J. 21, 81–87.
[5] Eder, W. and von Mutius, E. (2004) Hygiene hypothesis and endotoxin: what is the evidence. Curr. Opin. Allergy Clin. Immunol. 4, 113–117.
[6] Liu, A.H. and Mruphy, J.R. (2003) Hygiene hypothesis: fact or fiction?. J. Allergy Clin. Immunol. 111, 471–474.
[7] Herz, U., Lacy, P., Renz, H. and Erb, K. (2000) The influence of infections on the development and severity of allergic disorders. Curr. Opin. Immunol. 12, 632–640.
[8] Kim, D.S. and Darke-Lee, A.B. (2003) Infection, allergy and the hygiene hypothesis: historical perspective. J. Laryngol. Otol. 117, 946–950.
[9] Bjorksten, B., Sepp, E., Julge, K., Voor, T. and Mikelsaar, M. (2001) Allergy development and the intestinal microflora during the first year of life. J. Allergy Clin. Immunol. 108, 516–520.
[10] Fuller, R. (1989) Probiotics in man and animals. J. Appl. Bacteriol. 66, 365–378.
[11] Matsuzaki, T., Yamazaki, R., Hashimoto, S. and Yokokura, T. (1998) The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. J. Dairy Sci. 81, 48–53.
[12] Martinez, F.D. and Holt, P.G. (1999) Role of microbial burden in aetiology of allergy and asthma. Lancet 354, 12–15.
[13] Shida, K., Makino, K., Morishita, A., Takamizawa, K., Hachimura, S., Ametani, A., Sato, T., Kumagai, Y., Habu, S. and Kaminogawa, S. (1998) *Lactobacillus casei* inhibits antigen induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. Int. Arch. Allergy Immunol. 115, 278–287.
[14] Isolauri, E., Arvola, T., Sütas, Y. and Salminen, S. (2000) Probiotics in the management of atopic eczema. Clin. Exp. Allergy 30, 1605–1610.
[15] Sanfilippo, L., Li, C.K., Seth, R., Balwin, T.J., Menozzi, M.G. and Mahida, Y.R. (2000) *Bacteroides fragilis* enterotoxin induces the expression of IL-8 and transforming growth factor-beta TGF-beta by human colonic epitherial cells. Clin. Exp. Immunol. 119, 456–463.
[16] Hansen, G., McIntire, J.J., Yeung, V.P., Berry, G., Thorbecke, G.J., Chen, L., DeKruyff, R.H. and Umetsu, D.T. (2000) CD4(+) T helper cells engineered to produce latent TGF-beta 1 reverse allergen induced airway hyper reactivity and inflammation. J. Clin. Invest. 105, 61–70.
[17] Kato, I., Yokokura, T. and Mutai, M. (1984) Augmentation of mouse natural killer cell activity by *Lactobacillus casei* and its surface antigens. Microbiol. Immunol. 27, 209–217.
[18] Kato, I., Yokokura, T. and Mutai, M. (1988) Correlation between increase in Ia-bearing macrophages and induction of T-cell dependent antitumor activity by *Lactobacillus casei* in mice. Cancer Immunol. Immunother. 26, 215–221.
[19] Park, S.Y., Ji, G.E., Ko, Y.T., Jung, H.K., Ustunol, Z. and Pestka, J.J. (1999) Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. Int. J. Food Microbiol. 46, 231–241.

[20] You, H.J. and Oh, D.K. (2004) Anticancerogenic effect of a novel chiro-inositol containing polysaccharide from *Bifidobacterium bifidum* BGN4. FEMS Microbiol. Lett. 240, 131–136.

[21] Kim, I.H., Park, M.S. and Ji, G.E. (2003) Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 cells. J. Microbiol. Biotechnol. 13, 276–281.

[22] Marinaro, M., Boyaka, P.N., Finkelman, F.D., Kiyono, H., Raymond, J.J. and McGhee, J.R. (1997) Oral but parenteral interleukin-12 redirects T helper 2-type responses to an oral vaccine without altering mucosal IgA responses. J. Exp. Med. 185, 415–427.

[23] Li, X.M., Serebrisky, D., Lee, S.Y., Huang, C.K., Bardina, L., Schofield, B.H., Stanley, J.S., Burks, A.W., Bannon, G.A. and Sampson, H.A. (2000) A murine model of peanut anaphylaxis: T and B cell responses to a major peanut allergen mimic human responses. J. Allergy Clin. Immunol. 106, 150–158.

[24] Knippels, M.J., van Wijk, F. and Penninks, A.H. (2004) Food allergy: what do we learn from animal model?. Curr. Opin. Allergy Clin. Immunol. 4, 205–209.

[25] Hessle, C., Andersson, B. and Word, A. (2000) Gram-positive bacteria are potent inducers of monocytic interlukin-12 (IL-12) while Gram-negative bacteria preferentially stimulate IL-12 production. Infect. Immun. 68, 3581–3586.

[26] Lycke, N., Severinson, E. and Strober, W. (1990) Cholera toxin acts synergistically with IL-4 to promote IgG1 switch differentiations. J. Immunol. 145, 3316–3324.

[27] Park, J.H., Gold, D.R., Spiegelman, D.L., Burge, H.A. and Milton, D.K. (2001) House dust endotoxin and wheeze in the first year of life. Am. J. Respir. Crit. Care. Med. 163, 322–328.

[28] Dabbagh, K., Dahl, M.E., Steprick-Biek, P. and Lewis, D.B. (2002) Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. J. Immunol. 168, 4254–4260.

Biotechnology Letters (2005) 27: 1361–1367
DOI 10.1007/s10529-005-3682-9

Timing of *Bifidobacterium* administration influences the development of allergy to ovalbumin in mice

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Received 7 April 2005; Revisions requested 28 April 2005; Revisions received 23 June 2005; Accepted 28 June 2005

Key words: *Bifidobacterium*, food allergy, immunoglobulin E

Abstract

C3H/HeJ mice were sensitized with ovalbumin (OVA) and choleratoxin (CT) for 5 weeks, and then *Bifidobacterium bifidum* BGN4 was administered continuously for 7 weeks, starting 2 weeks before (pre-treatment group) and 2 weeks after (post-treatment group) the initial sensitization. After sensitization, the OVA-induced (sham group) mice showed growth inhibition and had scab-covered tails which was associated with serum levels of 9887 ± 175 ng OVA-specific IgE/ml and 758 ± 525 ng IgG1/ml. The sera of the pre-treatment group had 4805 ± 245 ng OVA-specific IgE/ml and 193 ± 87 ng IgG1/ml, as well as less severe tail symptoms. The sera of the post-treatment group had 5723 ± 207 ng OVA-specific IgE/ml but the IgG1 and IgG2a levels were the same as those of the sham group. In spleen cultures, both pre-treatment and post-treatment increased the levels of IFN-γ but decreased the levels of IL-6 and IL-18. Taken together, the *in vivo* and *in vitro* results show that treatment with *Bifidobacterium* before OVA sensitization suppresses or modulates the allergic response more effectively than treatment with Bifidobacterium following OVA sensitization.

Introduction

Food allergy is characterized by an abnormal immunologic reactivity to food components. It often begins in the first 1–2 years of life with the process of sensitization in which the immune system responds to specific food proteins, most often with the development of allergen-specific immunoglobulin E (IgE) (Matsuda & Nakamura 1993). The hygiene hypothesis (Gavett *et al.* 1995, Kirjavainen *et al.* 1999) proposes that the Th2-biased immune systems of new-born infants (Martinez & Holt 1999) might be changed to the Th1 type by exposure to gut bacteria after birth. A recent study showed that the administration of probiotics induces anti-inflammatory, tolerogenic immune responses (Schultz *et al.* 2003). Among the diverse probiotics, *Bifidobacterium* is one of the most promising, since it is the most predominant bacterium in infants (He *et al.* 2001). On the other hand, the timing of exposure to antigens or infections is widely accepted to be of primary importance in determining the phenotype of Th1 cell responses in neonates (Hosono *et al.* 1997, Holt & Sly 2002).

In the present study, to investigate the effect of timing of the administration of the probiotic bacteria, *Bifidobacterium* was administered to ovalbumin-induced mice at different time schedules. *Bifidobacterium bifidum* BGN4, which lowers the production of allergy-related cytokines from mouse cells (Kim et *al.* 2005), was used. The results show that the administration of *Bifidobacterium* before OVA sensitization was more

efficient in blocking the allergic response than administration after OVA sensitization.

Materials and methods

Mice

Three-week-old female C3H/HeJ mice, 11–13 g, were sensitized at 5 weeks of age and each group included six mice of similar weight. Mice were allowed free access to water, and maintained on a 12:12 h light:dark cycle in an environmentally controlled animal chamber at 23 ± 1 °C and 55 ± 10% humidity. The animal experimentation guidelines of Seoul National University were followed.

Microorganisms

Bifidobacterium bifidum BGN4 (BGN4) was anaerobically cultured in MRS broth (Difco). After centrifugation (4000×*g*, 40 min, 4 °C), bacteria were dried by lyophilization and mixed with mouse food (0.2% w/w). The total cell number of BGN4 was 3×10^9 c.f.u./g. The daily intake of diet for each mouse was about 3.0 g and the amount of bacteria intake for each mouse was approx. 6 mg.

Intragastric antigen sensitization and treatment

Mice were deprived of diet for 2 h preceding the oral sensitization. Sensitization was performed by intragastric administration of 50 μg ovalbumin (OVA, Sigma) with 10 μg cholera toxin (CT, Sigma) using a stainless blunt feeding needle. Four groups of mice were used in this study (Figure 1).

Measurement of OVA-specific and total immunglobulins in serum and in fecal samples

To determine serum antibody responses, tail vein blood was obtained biweekly following initial sensitization. Sera were collected and stored at −80 °C. Extracts of fecal pellets were prepared as described by Marinaro *et al.* (1997). Antibody levels were determined by ELISA according to the manufacture's protocol (Pharmingen).

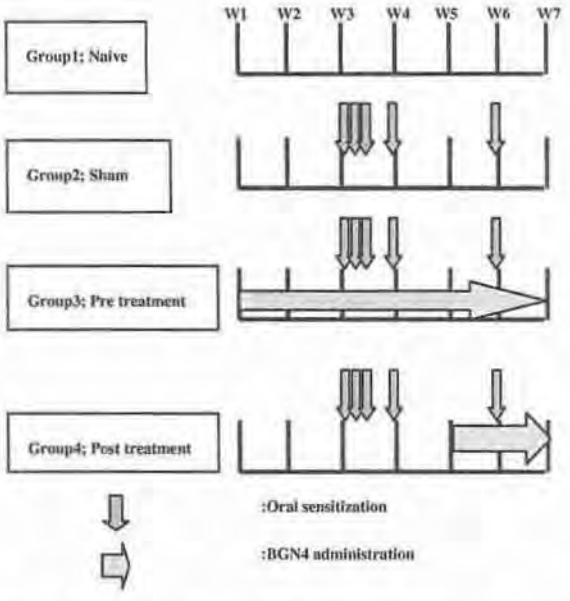

Fig. 1. Experimental protocol: Intragastric ovalbumin sensitization and BGN4 administration. Mice were sensitized at weeks 3, 4, and 6 with ovalbumin and choleratoxin. Mice were fed 0.2% of lyophilized *Bifidobacterium bifidum* BGN4 in diet pellets starting 2 weeks before (group 3) or after (group 4) the initial sensitization until sacrifice. The naive mice in group 1 received phosphate buffer saline (pH 7.2) instead of ovalbumin and choleratoxin as a negative control. Mice in group 2 were administered cornstarch instead of bacteria powder as a sham treatment. Each group included six mice.

Measurement of IL-6, IL-18, and IFN-γ levels in spleen

Spleens were isolated from mice from each group ($n=6$) at week 7. Spleen cells from mice were separated using glass slides and a 200 gauge stainless mesh. Cells were cultured in RPMI medium and stimulated with concanavalin A (10 μg/ml) for 48 h in 24-well flat bottom plates at a density of 5×10^6 cells/ml under 5% CO_2. ELISA kits (Pharmingen) were used for detecting the various cytokines.

Assessment of hypersensitivity reactions

Allergic symptoms were evaluated after sacrifice utilizing a scoring system: 0, no symptoms; 1, puffiness of the tail; 2, 1–2 scabs on the tail; 3, 3–4 scabs on the tail; 4, 5–6 scabs on the tail; 5, more than 7 scabs on the tail. The scoring of the symptoms was evaluated by 10 individuals who were unaware of sample identity in a blind manner.

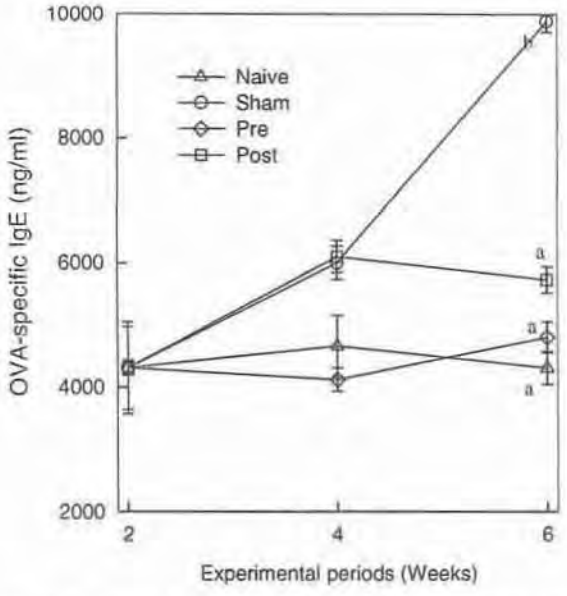

Fig. 2. Effect of *Bifidobacterium* BGN4 administration on production of ovalbumin specific IgE in serum from ovalbumin-sensitized mice. Sera from all groups of mice were obtained biweekly following initial ovalbumin sensitization. IgE levels were determined by ELISA.

Statistical analysis

All data are presented as the mean ± standard error of mean (SEM), indicated by bars in the figures. Data were analyzed using SAS (Release 8.01, O, USA). Differences between immunoglobulin and cytokine levels in the groups were analyzed by ANOVA followed by Duncan's multiple range test for multiple comparisons. p values < 0.05 were considered significant.

Results and discussion

Effect of BGN4 on IgE production

The OVA-specific IgE levels in sera of each group are presented in Figure 2. Pre-treatment with BGN4 markedly inhibited the production of OVA-specific IgE at week 6 (Naive, 4310 ± 263 ng/ml; Sham, 9887 ± 175 ng/ml; Pre, 4805 ± 245 ng/ml; Post, 5723 ± 207 ng/ml). Since IgE levels in the pre-treatment group did not increase compared with the sham group during the experimental period, pre-treatment with BGN4 would appear to block the induction of

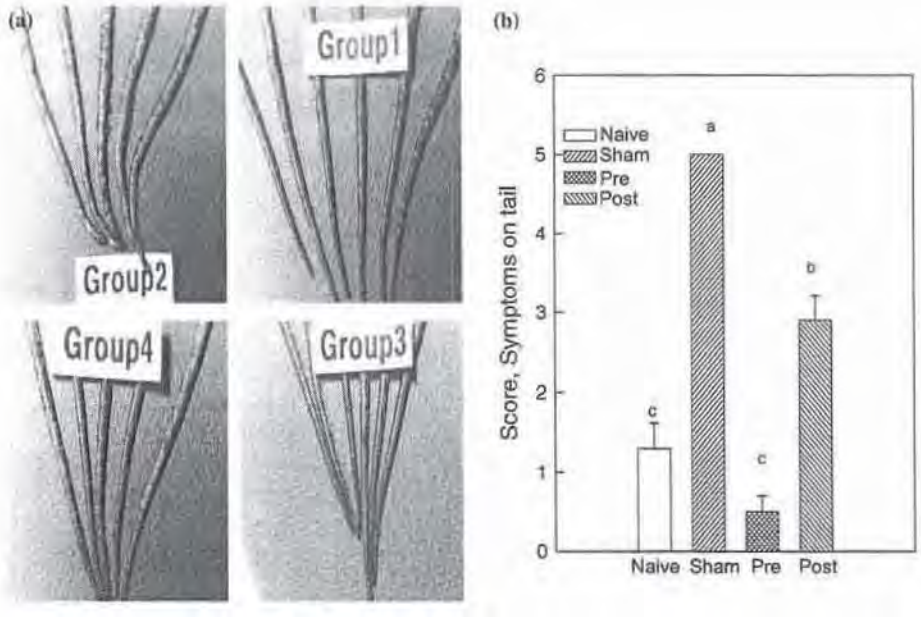

Fig. 3. Severity of tail allergy symptoms in ovalbumin-sensitized mice treated with *Bifidobacterium bifidum* BGN4 (BGN4) at week 7. Immunized mice showed marked tail bruising and scabs. A: Pictures of the tails from ovalbumin-sensitized mice treated with BGN4. Group 1: naive group; Group 2, sham group; Group 3, BGN4 pre-treatment group; Group 4, BGN4 post-treatment group. B: Severity of tail allergy symptoms in ovalbumin-sensitized mice was evaluated utilizing a scoring system in a blind manner.

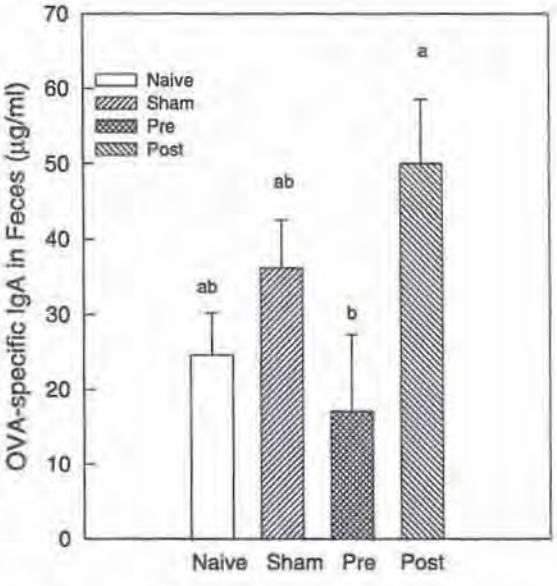

Fig. 4. Effect of *Bifidobacterium* on the production of ovalbumin specific IgA in fecal samples from ovalbumin-sensitized mice treated with BGN4. Fecal extracts were prepared from fresh fecal pellets of each group and ovalbumin-specific IgA levels were detected by ELISAs .

OVA-specific IgE entirely. On the other hand, treatment with BGN4 after initial sensitization reduced OVA-specific IgE levels less extensively.

Tail symptoms

After sensitization, sham mice started to scratch their tails, resulting in injury, including bleeding (Figure 3). The tail symptoms of the pre-treatment group were markedly reduced compared to the severe symptoms of the sham group at week 7, and those of the post-treatment group were decreased compared to those of the sham group, although the former still showed slight tail injuries. Therefore, the pre-treatment group showed the most effective attenuation of the tail injuries.

Ovalbumin specific mucosal IgA

The OVA-specific IgA level in the post-treatment group was not significantly different from that in the sham and naive groups at week 7 (Figure 4). However, the OVA-specific IgA level in the pre-treatment group was decreased compared to that of the sham group (Naive, 24.6 ± 5.6 μg/ml; Sham, 36.2 ± 6.4 μg/ml; Pre, 17.1 ± 10.2 μg/ml; Post, 54.6 ± 8.5 μg/ml, respectively).

Levels of IgG1 and IgG2a in sera

The levels of OVA-specific IgG1 in the post-treatment group were different from those of the naive group and pre-treatment group at week 7 (Figure 5a). On the other hand, only the levels of OVA-specific IgG2a in the pre-treatment group were different from those of the other groups. (Figure 5b). However, there were no differences in the levels of total IgG1 and IgG2a between the groups (Figure 5c, d).

The decreased levels of OVA-specific IgA, IgG1 and IgG2a in the pre-treatment groups are consistent with those of Mandic *et al.* (2004) who reported that the levels of IgG and IgA are high in allergic asthma patients compared to healthy children.

Table 1. Body weights (g) of ovalbumin-sensitized mice fed a diet containing (w/w) 0.2% of BGN4 lyophilized powder before or after initial sensitization for 7 weeks.[1]

Groups	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Naïve	17.2 ± 0.6^{b}	18.8 ± 0.5^{a}	19.1 ± 0.8^{a}	22.4 ± 0.6^{a}	22.9 ± 0.7^{a}	23.3 ± 1.1^{a}
Sham	18.8 ± 0.4^{b}	18.5 ± 0.3^{ab}	19.9 ± 0.3^{a}	20.5 ± 0.5^{b}	21.2 ± 0.4^{b}	21.0 ± 0.6^{b}
Pre	19.5 ± 0.3^{a}	19.7 ± 0.4^{a}	20.9 ± 0.3^{a}	21.2 ± 0.2^{ab}	21.8 ± 0.4^{ab}	22.9 ± 0.4^{a}
Post	16.9 ± 0.3^{b}	19.0 ± 0.2^{a}	20.6 ± 0.4^{a}	21.5 ± 0.3^{ab}	21.2 ± 0.5^{b}	21.9 ± 0.1^{ab}

[1]Experimental diets:
Naive: 0.2% of corn starch.
Sham: 0.2% of corn starch.
Pre: 0.2% of *Bifidobacterium bifidum* BGN4 (3×10^{9} c.f.u./g) starting 2 weeks before initial sensitization.
Post: 0.2% of *Bifidobacterium bifidum* BGN4 (3×10^{9} c.f.u./g) starting 2 weeks after initial sensitization.
Values are given as mean ± SEM of six mice per group.
Different superscripts indicate significant differences determined by Duncan's multiple range test ($p < 0.05$).

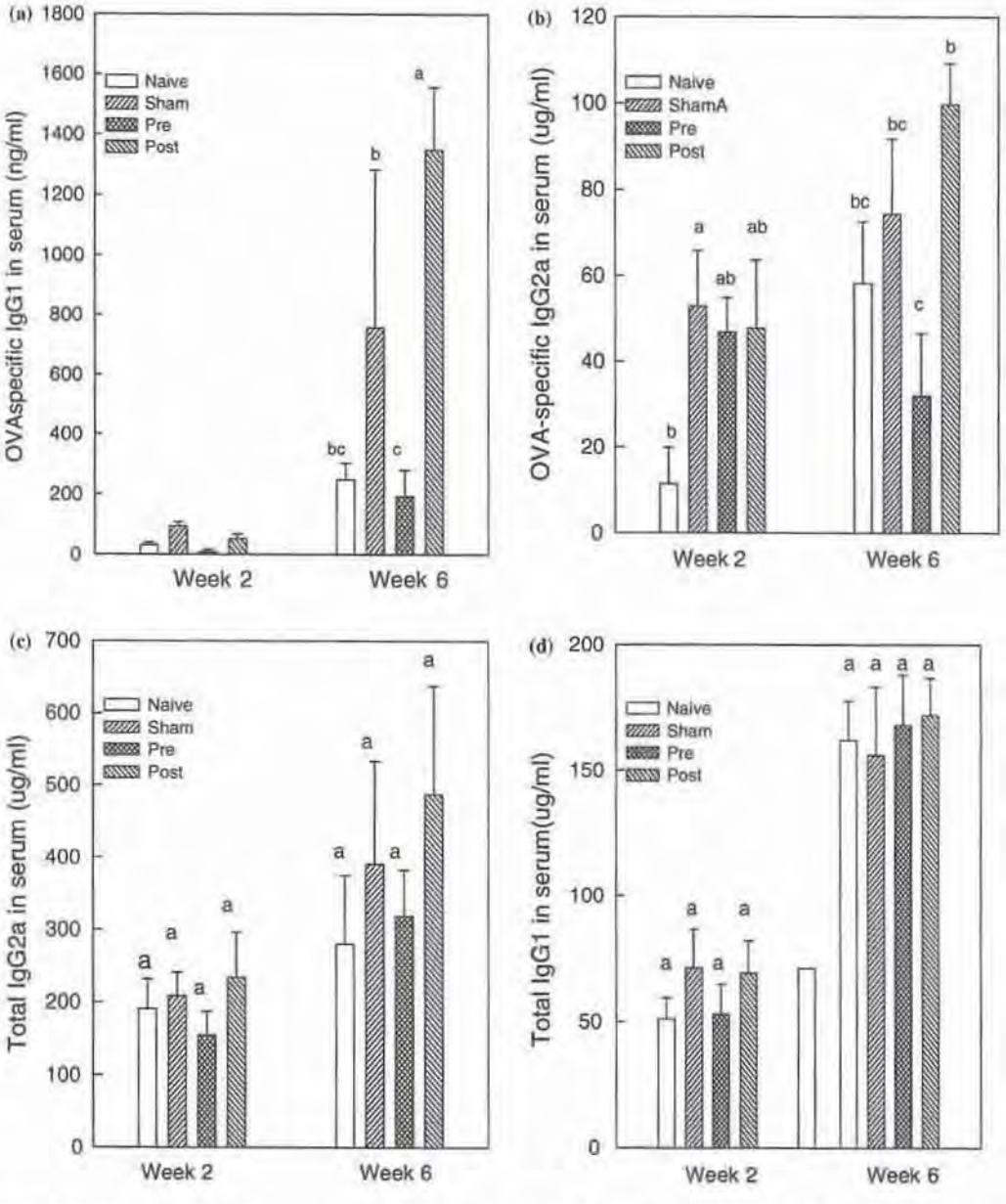

Fig. 5. Effect of *Bifidobacterium* on the production of ovalbumin-specific IgG1 and total IgG1 and ovalbumin-specific IgG2a and total IgG2a in sera from ovalbumin-sensitized mice treated with BGN4. Levels of antibodies were detected by ELISAs.

Levels of cytokines in spleen cultures

The levels of IFN-γ, IL-6, and IL-18 are given in Figure 6. In the *in vitro* spleen culture assay both pre-treatment and post-treatment with BGN4 markedly increased Th1 type cytokine IFN-γ and decreased Th2 type cytokines IL-6 and IL-18 compared with the sham group. Recent reports indicate that IL-18 can directly stimulate IL-4 production and histamine release from basophils (Yoshimoto *et al.* 1999), enhance IL-4 and IL-13 production from both NK and T cells in synergy with IL-2 (Leite-De-Moraes *et al.* 2001), and induce IgE expression by B cells (Yoshimoto *et al.* 2000).

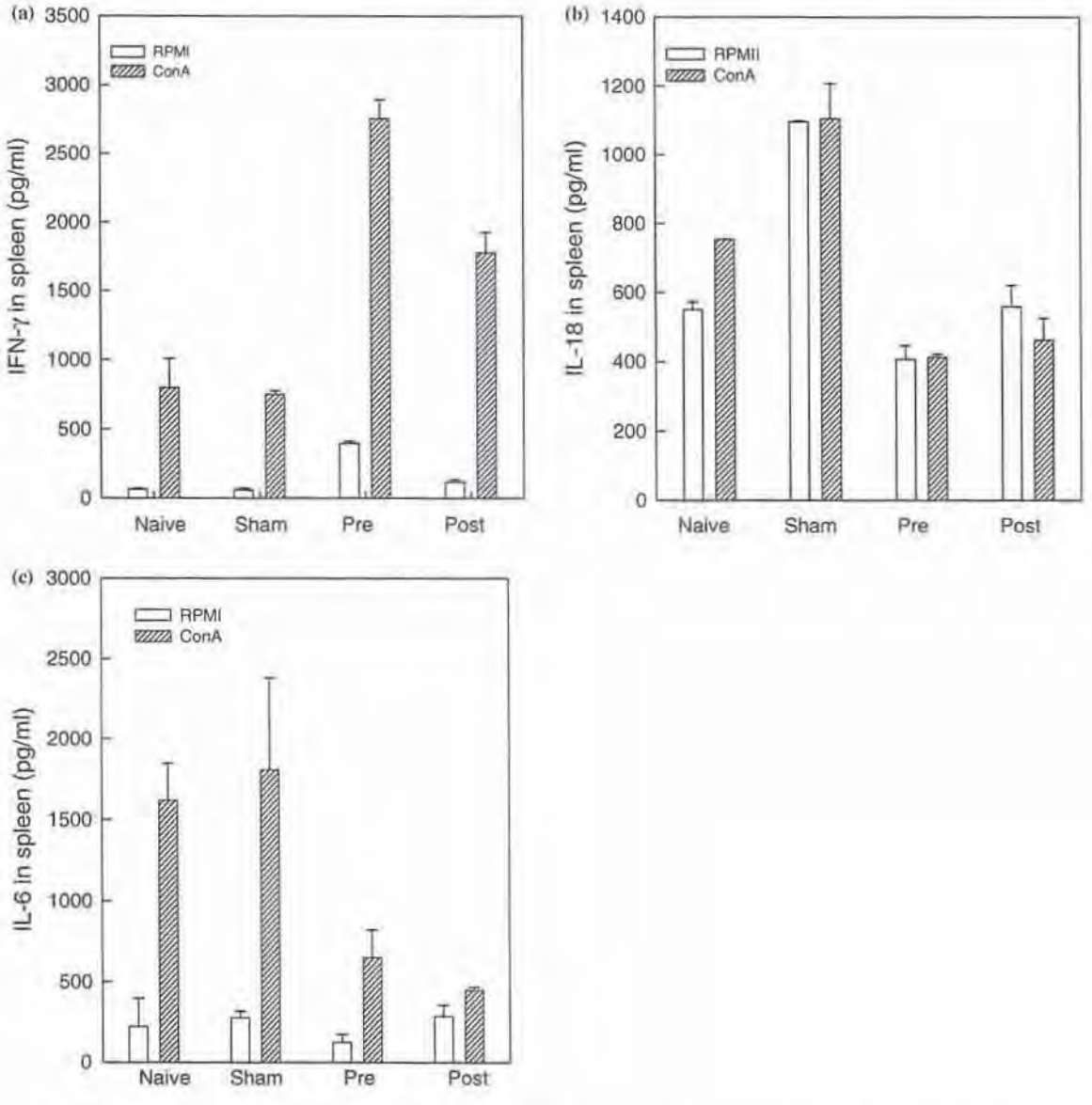

Fig. 6. Effect of *Bifidobacterium* on the production of IFN-γ, IL-6, IL-18, IgG1, and IgG2a from spleen cells of each group with BGN4 at week 7. Isolated spleen cells from different mouse groups were re-stimulated with concanavalin A (10 mg/ml).

Effect of BGN4 administration on body weight

The mean initial body weights did not differ significantly among the groups. Unlike the sham group, the pre-treatment group maintained similar body weights to the naive group during the experimental period (Table 1). BGN4 administration might decrease growth inhibition in the sham group.

Taken together, the *in vivo* and *in vitro* results show that pre-treatment with *Bifidobacterium* suppresses both the systemic and cellular allergic response, whereas post-treatment changes the cellular response in the spleen but only partially alters the systemic response.

In conclusion, these results demonstrate that oral administration of *Bifidobacterium* before

sensitization (or occurrence of allergy) profoundly prevents allergic response. However, the anti-allergenic effect of *Bifidobacterium* administered after outbreak of allergic disease is weaker than the effect of *Bifidobacterium* administered before administration of sensitization stimulus. Thus, intake of *Bifidobacterium* continuously from before the occurrence of allergic disease might be recommended to minimize allergic symptoms.

Acknowledgement

This work was supported by the Korean Ministry of Science and Technology (NRL, MI-0302-00-0098).

References

Gavett SH, O'hearn DJ, Li X, Huang S-K, Finkelman FD, Willis-Karp M (1995) Interleukin 12 inhibit antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med.* **182**: 1527–1536.

He F, Ouwehand AC, Isolauri E, Hashimoto H, Benno Y, Salminen S (2001) Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. *FEMS Immunol. Med. Microbiol.* **30**: 43–47.

Holt PG, Sly PD (2002) Interactions between RSV infection, asthma, and atopy, unraveling the complexities. *J. Exp. Med.* **18**: 1271–1275.

Hosono A, Lee J, Ametani A, Natsume M, Hirayama M, Adachi T, Kaminogawa S (1997) Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M 101-4. *Biosci. Biotech. Biochem.* **61**: 312–316.

Kim HY, Yang JH, Ji GE (2005) Effect of bifidobacteria on production of allergy-related cytokines from mouse cells. *J. Microbiol. Biotechnol.* **15**: 265–268.

Kirjavainen PV, Apostolou E, Salminen SJ, Isolauri E (1999) New aspects of probiotics-a novel approach in the management of food allergy. *Allergy* **54**: 909–915.

Leite-De-Moraes MC, Hameg A, Pacilio M, Koezuka Y, Taniguchi M, Van Kaer L, Schneider E, Dy M, Herbelin A (2001) IL-18 enhances IL-4 production by ligand-activated NKT lymphocytes: a pro-Th2 effect of IL-18 exerted through NKT cells. *J. Immunol.* **166**: 945–951.

Mandic Z, Marusic M, Boranic M (2004) Low levels of immunoglobulin A in children with intrinsic asthma: a possible protection against atopy. *Med. Hypotheses* **62**: 600–604.

Marinaro M, Boyaka PN, Finkelman FD, Kiyono H, Raymond HH, McGhee JR (1997) Oral but parenteral interleukin-12 redirects T helper 2-type responses to an oral vaccine without altering mucosal IgA responses. *J. Exp. Med.* **185**: 415–427.

Martinez FD, Holt PG (1999) Role of microbial burden in aetiology of allergy and asthma. *Lancet* **354**: 12–15.

Matsuda T, Nakamura R (1993) Molecular structure and immunological properties of food allergens. *Trends Food Sci. Technol.* **4**: 289–293.

Schultz M, Linde HJ, Lehn N, Zimmermann K, Grossmann J, Falk W, Scholmerich J (2003) Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers. *J. Dairy Res.* **70**: 165–173.

Yoshimoto T, Tsutsui H, Tominaga K, Hoshino K, Okamura H, Akira S, Paul WE, Nakanishi K (1999) IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proc. Natl. Acad. Sci. USA* **96**: 13962–13966.

Yoshimoto T, Mizutani H, Tsutsui H, Noben-Trauth N, Yamanaka K, Tanaka M, Izumi S, Okamura H, Paul WE, Nakanishi K (2000) IL-18 induction of IgE: dependence on CD4+ T cells, IL-4 and STAT6. *Nature. Immunol.* **1**: 132–137.

J. Microbiol. Biotechnol. (2006), **16**(7), –

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of Viability and Integrity of *Bifidobacterium* on Suppression of Allergy in Mice

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Received:
Accepted:

Abstract The effects of the cell viability and integrity of *Bifidobacterium* on suppression of allergy were investigated. C3H/HeJ mice were sensitized on weeks 3, 4, 6, and 8 with ovalbumin and choleratoxin to induce an allergic reaction. Mice fed 0.2% of live, disrupted, or heat-killed *Bifidobacterium bifidum* BGN4 in the pellets of their diet for 8 weeks starting 2 weeks before initial sensitization differentially suppressed the allergy response in terms of levels of IgE and IgG1 in their sera, and symptoms on their tails. Viable *Bifidobacterium* was more effective than disrupted or heat-killed cells in suppressing the allergy. Growth inhibition, which occurred in the sham group at week 4, did not occur in the treated groups. These results show that *Bifidobacterium* has a suppressive effect on the allergic response of mice, and that the viability and integrity of the *Bifidobacterium* is required for effective suppression in our experimental model.

Key words: Food allergy, IgE, *Bifidobacterium*

The beneficial effects of bifidobacteria on health have been widely studied. Yoshioka *et al.* [31] showed that bifidobacteria predominated in the feces of breast-fed infants, but that in the feces of bottle-fed infants, *Escherichia coli*, *Streptococcus faecalis*, and *Bacteroides* sp. were more frequent. Many beneficial effects of bifidobacteria have been reported such as its anticancer activity [5], lowering of plasma cholesterol [6], prevention of constipation [22], and stimulation of the immune system [29]. Bifidobacteria are also thought to protect against intestinal disorders. Hotta *et al.* [9] investigated the effects of administration of *Bifidobacterium* preparations on infant diarrhea, and Hattori *et al.* [8] reported that the administration of *Bifidobacterium* for 1 month to children with atopic dermatitis resulted in significant amelioration of their allergic symptoms, although they did not find any correlation between changes in fecal microflora and levels of allergic symptoms.

There have been only a few studies of the anti-allergic effects of lactic acid bacteria. Matsuzaki *et al.* [21] showed that oral administration of *Lactobacillus casei* Shirota inhibited IgE production. Moreover, administration of *L. casei* induced the formation of Th1 cell-associated cytokines, such as IFN-γ and IL-2, by spleen cells, but suppressed the production of Th2-associated cytokines such as IL-4, IL-5, IL-6, and IL-10. In addition, Shida *et al.* [30] showed that *L. casei* inhibited antigen-induced IgE by stimulating secretion of IL-12 by macrophages. The mechanism by which probiotics prevent allergic response is still unknown. One possible basis of the decrease of IgE-mediated allergic responses could be related to IL-12. Identification of the effective components or structural requirements of the probiotics should be of value in exploiting their allergy preventing potential. Several components of the Gram-positive bacterial cell wall, e.g., capsular polysaccharides, peptidoglycans, and lipoteichoic acids, stimulate the production of IFN-γ and TNF-α in murine cells, and of IFN-γ in human peripheral blood lymphocytes [4, 7, 15, 16, 26, 28].

In the present study, we characterized the immuno-regulatory effects of peroral administration of live, heat-killed, and disrupted cells of *Bifidobacterium bifidum* BGN4 in a mouse model of food allergy. Our aim was to identify an effective way of using *Bifidobacterium* to prevent the occurrence of food allergies.

Materials and Methods

Mice

Three-week-old female C3H/HeJ mice weighing 11–13 g were purchased from Japan SLC (Hamamatsu, Japan) and maintained on ovalbumin (OVA)-free chow. Sensitization

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of the mice was started at 5 weeks of age, and six mice were included in each group. The mice were kept in plastic cages, allowed free access to water, and maintained on a 12:12 h light:dark cycle in an environmentally-controlled animal chamber. Temperature and humidity were controlled at 23±1°C and 55±10%, respectively. The animal experimentation guidelines of Seoul National University were followed.

Microorganisms

B. bifidum BGN4 was isolated from the feces of Korean subjects, as described in a previous study [25]. *Bifidobacterium* and *Lactobacillus* were cultured anaerobically in Lactobacilli-MRS broth (Difco, Detroit, MI, U.S.A.) containing 0.05% L-cysteine (Sigma, St. Louis, MO, U.S.A.) at 37°C for 24 h. To prepare the mouse diets, live bacterial cells were collected by centrifugation (Hanil, Seoul, Korea) at 4,000 ×*g* for 40 min at 4°C, and washed twice with sterile phosphate-buffered saline. To prepare heat-killed and disrupted cells, respectively, the bacteria were exposed to 95°C for 30 min, or disrupted in a cell disrupter (Stansted Fluid Power, Essex, U.K.) for 10 min. The bacterial preparations were then lyophilized (Ilshin, Seoul, Korea) and used as dietary components.

Intragastric Antigen Sensitization and Treatment

Mice were starved for 2 h preceding oral sensitization. Sensitization was performed by intragastric (ig) administration of OVA (50 μg) with 10 μg of cholera toxin (CT) on days 0, 1, 2, 7, and 21, using a blunt stainless steel feeding needle. OVA (Sigma, St. Louis, MO, U.S.A.) served as the antigen. CT and concanavalin A (Con A) were purchased from Sigma (St. Louis, MO, U.S.A.). Five groups of mice were used in this study (Fig. 1). The mice in groups 2 to 5 were gavaged with 0.2 ml phosphate buffer saline solution (PBS, pH 7.2) containing OVA and CT, whereas the mice in the naive group (group 1) were gavaged with PBS without OVA and CT. Mice in groups 2 to 5 were subjected to the same OVA sensitization, and the mice in groups 3 to 5 were administered bacterial powder, whereas those in group 2 acted as sham controls and received OVA and CT but no bacteria. The bacteria-treated mice were fed 0.2% of lyophilized *B. bifidum* BGN4 (BGN4) in the form of diet pellets of live cells, disrupted cells, or heat-killed cells. They were fed the experimental bacterial powders for 7 weeks, starting 2 weeks before the initial sensitization until they were killed. To assess serum antibody responses, tail vein blood was collected weekly after the initial sensitization. Sera were stored at −80°C.

Measurement of Serum OVA-Specific IgE, IgG1, IgG2a, and Total IgE from Sera

Sera were collected weekly from tail veins and stored at −80°C. Levels of OVA-specific IgE, IgG1, or IgG2a were measured by ELISA. Briefly, Nunc-Immuno-Maxisorp plates

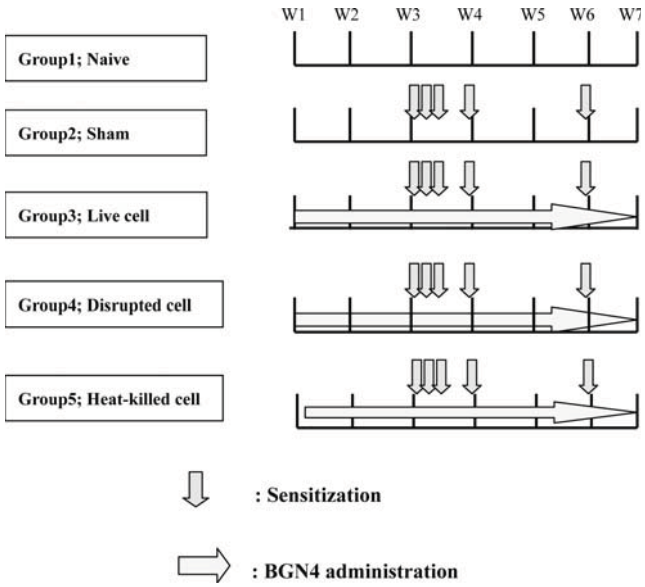

Fig. 1. Experimental protocol for intragastric ovalbumin sensitization and bacterial administration.
Mice were sensitized on weeks 3, 4, and 6 with ovalbumin and cholera toxin. Mice in group 3, group 4, and group 5 were fed 0.2% lyophilized live cells, disrupted cells, or heat-killed cells of *Bifidobacterium bifidum* BGN4, respectively, in the pellets of their diet for 8 weeks, starting 2 weeks before initial sensitization until sacrifice. The naive mice in group 1 were administered PBS buffer instead of ovalbumin and cholera toxin, and the mice in group 2 (sham) were administered corn starch instead of bacterial powder. Each group consisted of 6 mice.

(Nunc, Roskilde, Denmark) were coated with 5 μg/ml of OVA in coating buffer, pH 9.6 (Sigma, St. Louis, MO, U.S.A.), overnight at 4°C. The plates were blocked and washed. Samples were added to the plates and incubated overnight at 4°C. The plates were washed again, and biotinylated rat anti-mouse IgE, IgG1, or IgG2a monoclonal antibodies (2 μg/ml) were added for 1 h at room temperature to detect OVA-specific IgE, IgG1, and IgG2a, respectively. After incubation with horseradish peroxidase (HRP), (Pharmingen, San Diego, CA, U.S.A.), the reactions were developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Fluka, Neu-Ulm, Switzerland) for 30 min at room temperature. The color reactions were stopped with 6 N H_2SO_4 and read at 450 nm. Levels of IgE, IgG1, or IgG2a were calculated using reference curves generated with standards of total mouse IgE, IgG1, and IgG2a, respectively. Total IgE levels in sera were also determined by ELISAs. The plates were coated with 2 μg/ml of rat monoclonal anti-mouse IgE. Serial dilutions of sera were added, followed by 100 μl of a biotinylated rat monoclonal anti-mouse IgE. The subsequent steps were as described above. All of the antibodies used in this study were purchased from Pharmingen.

Measurement of OVA-Specific and Total Fecal IgA

Extracts of fecal pellets were prepared as described by Marinaro *et al.* [20]. In brief, 100 mg of pellet was mixed

with 1 ml of PBS containing 0.1% NaN_3 and incubated at 4°C for 2 h. The fecal pellets were mixed vigorously in PBS for 10 min. After centrifugation (4,000 ×*g*, 20 min), the supernatants were collected and stored at −70°C. To assay OVA-specific IgA, plates were coated with 5 μg/ml of OVA in coating buffer. After washing and blocking, 100 μl of fecal extracts was added to individual wells and incubated overnight at 4°C. The plates were then washed, and biotinylated rat anti-mouse IgA monoclonal antibody (2 μg/ml) was added and incubated for 1 h at room temperature. After washing, streptavidin-peroxidase (Sigma) was added for 1 h at room temperature. The reactions were developed with TMB for 30 min at room temperature, stopped with 6 N H_2SO_4 and read at 450 nm. Equivalent levels of IgA were calculated using a reference curve generated with mouse total IgA as standard. To measure total IgA, plates were coated with rat anti-mouse IgA capture antibodies (2 μg/ml) in coating buffer, blocked and washed as described above, and fecal extracts (1:50 dilutions) were added to the plates and incubated overnight at 4°C. The plates were washed and 100 μl of biotinylated rat anti-mouse IgA was added to each well. The subsequent steps were as described above. IgA levels were calculated from a reference curve generated with mouse total IgA.

Assessment of Hypersensitivity Reactions

Allergic symptoms were evaluated after sacrifice using the scoring system: 0, no symptoms; 1, puffiness of the tail; 2, 1-2 scabs on the tail; 3, 3-4 scabs on the tail; 4, 5-6 scabs on the tail; 5, more than 7 scabs on tail. Symptoms were scored blind manner, and the scores were evaluated by ten individuals unaware of sample identities.

Statistical Analysis

All data are presented as the mean±standard error of mean (SEM), as indicated by the bars. Data were analyzed using SAS (Release 8.01, NC, U.S.A.). Differences between immunoglobulin and cytokine levels were analyzed by ANOVA followed by Duncan's multiple range tests for multiple comparisons. *P* values <0.05 were considered significant.

RESULTS

Effect of BGN4 on IgE Production

To monitor possible effects of the BGN4 fractions, sera were obtained from each group. The OVA-specific IgE in the sera of each group are presented in Fig. 2. Levels of OVA-specific IgE and total IgE in the sham group were significantly higher than those in the naive and BGN4-treated groups at week 6.

Symptoms on Tails

Mice sensitized with OVA and CT showed some injuries on their tails. After the administration of OVA and CT, the sham mice started to scratch their tails, and caused some injury including bleeding (Fig. 3). No injuries and scratches were observed in the group treated with live cells, whose symptom scores did not differ from those of the naive group. The symptom scores on the tails of the group treated with disrupted cells were lower than in the sham group but higher than in the other groups, and significantly higher than in the naive group. The symptom scores in the group treated with heat-killed cells were

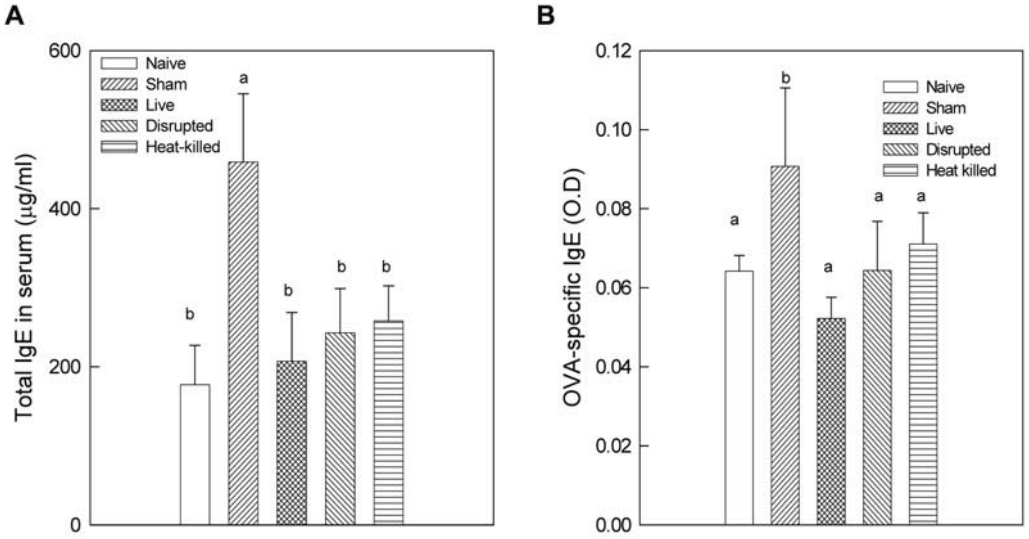

Fig. 2. Effect of administration of bacteria on the levels of total IgE and ovalbumin-specific IgE in sera from ovalbumin-sensitized mice.

IgE levels were determined by ELISAs. Total IgE and ovalbumin-specific IgE levels were determined on week 6. Data are means±SEM of 6 mice per group. Different letters indicate significant differences in Duncan's multiple range tests ($p<0.05$).

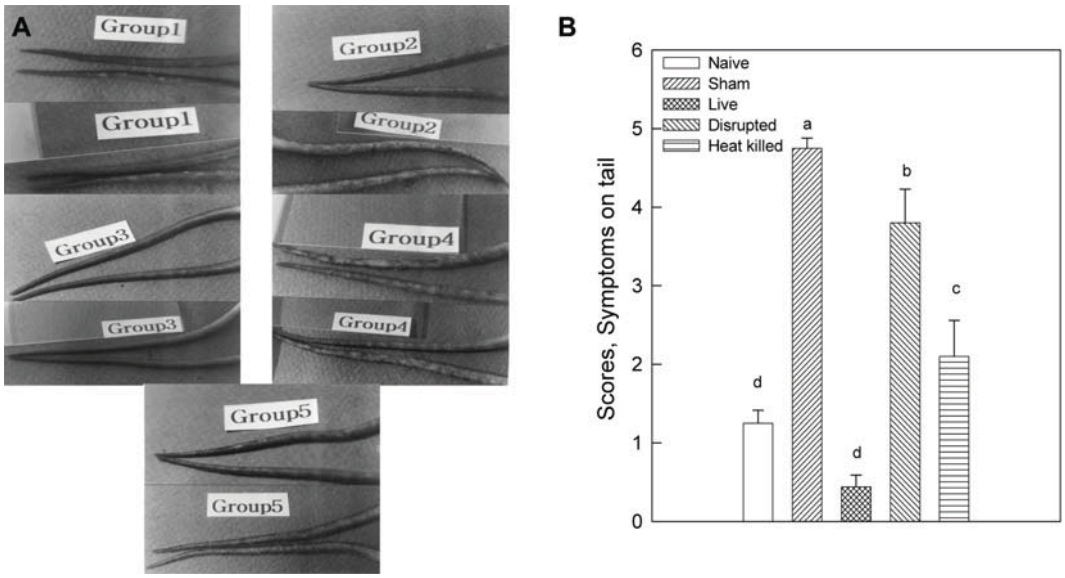

Fig. 3. Severity of allergic symptoms on the tails of ovalbumin-sensitized mice treated with various components of *Bifidobacterium bifidum* BGN4.
The immunized mice showed marked tail bruising and scabs. **A**. The tails of OVA-sensitized mice treated with bacteria. Group 1, naive; Group 2, sham; Group 3, treated with live BGN4; Group 4, treated with disrupted BGN4; Group 5, treated with heat-killed BGN4. **B**. Severity of allergic symptoms on the tails of OVA-sensitized mice. Data are means±SEM. Different letters indicate significant differences in Duncan's multiple range tests ($p<0.05$).

lower than in the sham group or the group treated with disrupted cells (Naive, 1.3±0.2; Sham, 4.8±0.1; Live, 0.4±0.2; Disrupted, 3.8±0.4; Heat-killed, 2.1±0.5, respectively).

OVA-Specific Mucosal IgA

We evaluated OVA-specific mucosal IgA and total IgA levels in extracts of fecal samples collected on week 7 (Fig. 4). OVA-specific IgA in the BGN4-treated groups was significantly different from that in the sham or naive groups. The levels of OVA-specific IgA in the live cell, disrupted cell, and heat-killed cell groups were markedly lower than those in the sham group. There were no differences in total IgA levels between the five experimental groups (OVA-specific IgA; Naive, 291.8±29.9 g/ml; Sham, 210.6±34.5 g/ml; Live, 56.2±4.8 g/ml; Disrupted, 89.2±9.2 g/ml; Heat-killed, 60.8±12.9 g/ml, respectively).

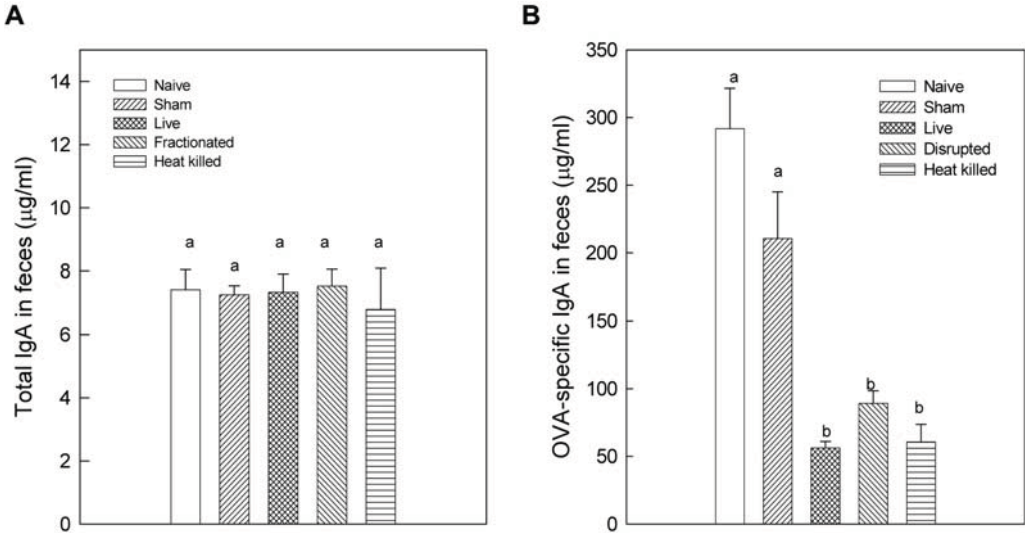

Fig. 4. Effect of *Bifidobacterium* on the production of total IgA (**A**) and ovalbumin-specific IgA (**B**) in fecal samples from ovalbumin-sensitized mice and bifidobacteria-treated mice.
Fresh fecal pellets from each group were collected on week 7. Fecal extracts were prepared and ovalbumin-specific IgA levels detected by ELISA. Data are means±SEM of 6 mice per group. Different letters indicate significant differences in Duncan's multiple range tests ($p<0.05$).

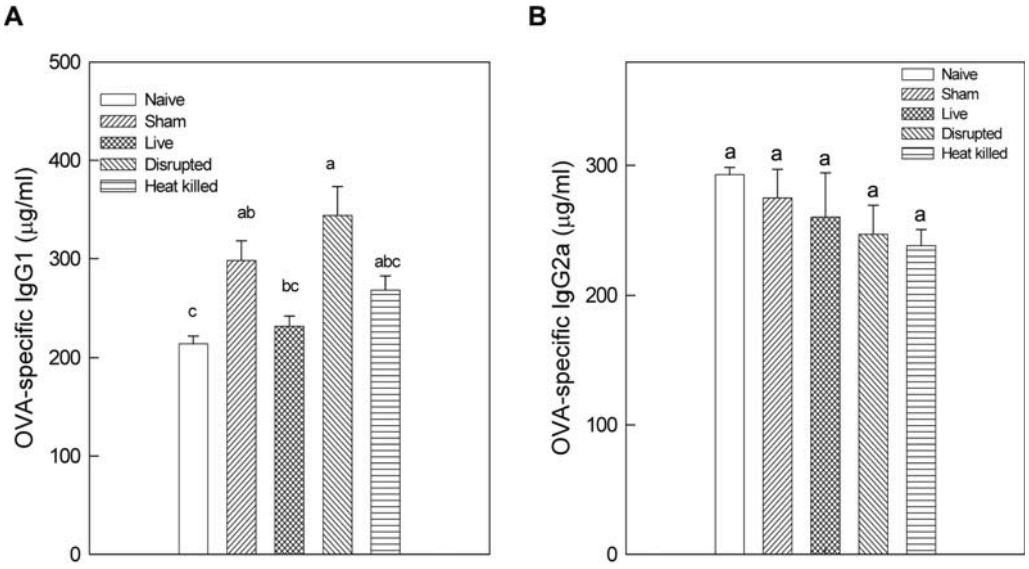

Fig. 5. Effect of *Bifidobacterium* on the production of ovalbumin-specific IgG1 (**A**) and ovalbumin-specific IgG2a (**B**) in sera from ovalbumin-sensitized and bifidobacteria-treated mice.
Levels of antibody were detected by ELISAs. Data are means±SEM of 6 mice per group. Different letters indicate significant differences in Duncan's multiple range tests ($p<0.05$).

IgG1 and IgG2a Levels in Sera

The levels of OVA-specific IgG1 in the sera of the sham group on week 6 were significantly higher than those of the naive group. The levels of OVA-specific IgG1 in the live cell or heat-killed cell groups were as low as in the naive group. The levels of OVA-specific IgG1 in the disrupted cell group were higher than those in the naive group. On the other hand, the levels of OVA-specific IgG2a in the sham group did not differ from those of the naive group. There were again no significant differences between the five experimental groups (Fig. 5).

Effect of BGN4 Administration on Body Weight

Mean body weights did not differ between the five experimental groups up to week 3 (Table 1). However, the body weights of the sham group were lower than those of the naive group on week 4, whereas no growth inhibition was observed in the *Bifidobacterium* treated groups over the experimental period.

DISCUSSION

In the present study allergic responses were suppressed in mice administered with *Bifidobacterium*. The suppressive effect of live *Bifidobacterium* was higher than that of heat-killed or disrupted cells. The suppressive effect may be due to specific components of the administered *Bifidobacterium*.

It has been shown in several studies that the composition of the microflora differs between healthy and allergic infants. *Bifidobacterium* content was low in allergic infants, and colonization with *Staphylococcus aureus* and enterobacteria was high [1]. In other research, atopic infants had more clostridia and fewer bifidobacteria than healthy infants [11]. These studies suggested that the microflora affect immune responses in allergic disease.

The gastrointestinal tract is a highly complex ecosystem composed of host cells, nutrients, and microflora. This ecosystem plays an important role in nutrition, physiology, and regulation of the immune system. It is known that bacteria in the microflora communicate with each other by

Table 1. Body weight of ovalbumin-sensitized mice and bifidobacteria-treated mice.

Groups	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Naive	15.1±0.2^{a}	18.6±0.2^{a}	20.2±0.3^{a}	20.2±0.3^{ab}	22.1±0.4^{a}	22.7±0.4^{a}
Sham	14.8±0.3^{a}	18.3±0.3^{a}	17.4±0.3^{c}	19.6±0.3^{b}	20.6±0.9^{b}	20.8±0.4^{b}
Live	14.7±0.2^{a}	17.8±0.3^{a}	18.4±0.3^{b}	20.1±0.4^{ab}	20.5±0.4^{b}	21.0±0.4^{b}
Disrupted	16.9±0.3^{a}	19.0±0.2^{a}	20.6±0.4^{b}	21.5±0.3^{b}	21.2±0.5^{b}	21.9±0.1^{b}
Heat-killed	15.3±0.3^{a}	18.6±0.4^{a}	20.1±0.3^{a}	21.3±0.4^{a}	21.8±1.6^{ab}	22.6±0.6^{a}

Values are means±SEM of 6 mice per group.
Different superscripts indicate significant differences as a result of Duncan's multiple range tests ($p<0.05$).

means of signals or antibiotic molecules, and this contributes to maintaining a beneficial ecosystem in the gastrointestinal tract. Microflora are influenced by dietary composition, age, probiotics, and drugs [2]. In order to affect the immune system, a microorganism must activate the lymphoid cells of the gastrointestinal lymphoid tissue [19]. Probiotics have been orally administered with the aim of affecting the microflora. However, bacteria may be damaged by stomach acids, bile salts, or pancreatic enzymes during their passage through the stomach and small intestine. In consequence, some of bacteria will reach the colon in the form of fragments or dead cells, which might still have immunomodulatory effects. Indeed, it is well known that immunomodulatory effects on microflora are related to interactions between the host immune system and bacterial components such as the lipopolysaccharide of Gram-negative bacteria, or lipoteichoic acid of Gram-positive bacteria. Namba *et al.* [23] showed that lysozyme enhanced the adjuvant effect of bacterial cell walls, and they suggested that bacteria are usually broken up enzymatically in the human intestinal tract, and that some components of the cells might be converted in the process into a more immunologically active form. These ideas have been supported by a report that after being fragmented by ozone, bacteria still had an immunomodulatory effect [10]. Therefore, we anticipated that disrupted cells might be effective in suppressing the allergy response. However, in the present study, disrupted cells had a weaker suppressive effect than live or heat-killed cells. The reduced production of OVA-specific IgE and IgA may have resulted from tight control of the Th2 cells that recognize OVA-antigen. The relatively greater reduction in IgG1 than in IgG2 suggests that the live bacteria shifted the balance between Th1 and Th2 toward Th1-type immunity. Although the active components of bacterial cells on allergy are not fully understood, mechanical disruption may damage some of the effective components such that their suppressive effect on OVA-induced allergy is related to their integrity.

In conclusion, the present study shows that viable and intact *Bifidobacterium* are more effective than disrupted or heat-killed cells in modulating the allergic response in mice, after oral administration.

Acknowledgment

This work was supported by the Korean Ministry of Science and Technology (NRL, MI-0302-00-0098).

REFERENCES

1. Bjorksten, B., P. Naaber, E. Sepp, and M. Mikelsaar. 1999. The intestinal microflora in allergic Estonian and Swedish 2-year old children. *Clin. Exp. Allergy* **29:** 342–346.
2. Bourlioux, P., B. Koletzko, F. Guarner, and V. Braesco. 2002. The intestine and its microflora are partners for the protection of the host: Report on the Danone Symposium "The intelligent intestine," held in Paris. *Am. J. Clin. Nutr.* **78:** 675–683.
3. Cleveland, M. G., J. D. Gorham, T. L. Murphy, E. Tuomanen, and K. M. Murphy. 1996. Lipoteichoic acid preparations of Gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Infect. Immun.* **64:** 1906–1912.
4. De Simone, C., R. Vesely, B. B. Salvadori, and E. Jirillo. 1993. The role of probiotics in modulation of the immune system in man and in animals. *Int. J. Immunother.* **9:** 23–28.
5. Glen, E. H. and R. S. Lambrecht. 1993. Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid producing bacteria. *J. Dairy Sci.* **76:** 2485–2492.
6. Goldin, B. R., A. H. Lichtenstein, and S. L. Gorbach. 1988. The role of the intestinal flora, pp. 500–512. *In* Shils, M. E. and V. R. Young (eds.), *Modern Nutrition in Health and Disease*. LFA and FEBIGER, Philadelphia.
7. Halpern, G. M., K. G. Vruwink, J. Van De Water, C. L. Keen, and M. E. Gershwin. 1991. Influence of long-term yoghurt consumption in young adults. *Int. J. Immunother.* **7:** 205–210.
8. Hattori, K., A. Yamamoto, M. Sasai, S. Taniuchi, T. Kojima, Y. Kobayashi, H. Iwamoto, K. Namba, and T. Yaeshima. 2003. Effects of administration of bifidobacteria on fecal microflora and clinical symptoms in infants with atopic dermatitis. *Arerugi* **52:** 20–30.
9. Hotta, M., Y. Saito, S. Iwata, N. Yamashita, K. Sunakawa, T. Olikawa, R. Tanaka, K. Watanabe, H. Takayama, M. Yajima, S. Kekiguchi, S. Arai, T. Sakurai, and M. Mutai. 1987. Clinical effects of *Bifidobacterium* preparations on pediatric intractable diarrhea. *Keio J. Med.* **36:** 298–314.
10. Kalka-Moll, W. M., A. O. Tzianabos, Y. Wang, V. J. Carey, R. W. Finberg, A. B. Onderdonk, and D. L. Kasper. 2000. Effect of molecular size on the ability of zwitterionic polysaccharides to stimulate cellular immunity. *J. Immunol.* **164:** 719–724.
11. Kalliomaki, M., P. Kirjavainen, E. Eerola, P. Kero, S. Salminen, and E. Isolauri. 2001. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J. Allergy Clin. Immunol.* **107:** 129–134.
12. Kim, H. Y., J. H. Yang, and G. E. Ji. 2005. Effect of bifidobacteria on production of allergy-related cytokines from mouse spleen cells. *J. Microbiol. Biotechnol.* **15:** 265–268.
13. Kim, I. H., M. S. Park, and G. E. Ji. 2003. Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 cells. *J. Microbiol. Biotechnol.* **13:** 276–281.
14. Kirjavainen, P. V., E. Apostolou, S. J. Salminen, and E. Isolauri. 1999. New aspects of probiotics - a novel approach in the management of food allergy. *Allergy* **54:** 909–915.
15. Kitazawa, H., K. Matsumura, T. Itoh, and T. Yamaguchi. 1992. Interferon induction in murine peritoneal macrophage by stimulation with *Lactobacillus acidophilus*. *Microbiol. Immunol.* **36:** 311–315.

16. Kitazawa, H., Y. Tomioka, K. Matsumura, H. Aso, M. Mizugaki, T. Itoh, and T. Yamaguchi. 1994. Expression of mRNA encoding IFN alpha in macrophages stimulated with *Lactobacillus gasseri*. *FEMS Microbiol. Lett*. **120:** 315–322.
17. Kubin, M., J. M. Chow, and G. Trinchieri. 1994. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor-α, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. *Blood* **83:** 1847–1855.
18. Lee, B. H. and G. E. Ji. 2005. Effect of *Bifidobacterium* cell fractions on IL-6 production in RAW 264.7 macrophage cells. *J. Microbiol. Biotechnol*. **15:** 740–744.
19. Madara, J. L. 1997. The chameleon within: Improving antigen delivery. *Science* **277:** 910–911.
20. Marinaro, M., P. N. Boyaka, F. D. Finkelman, H. Kiyono, R. J. JJackson, E. Jirillo, and J. R. McGhee. 1997. Oral but not parenteral interleukin-12 redirects T helper 2-type responses to an oral vaccine without altering mucosal IgA responses. *J. Exp. Med*. **185:** 415–427.
21. Matsuzaki, T., R. Yamazaki, S. Hashimoto, and T. Yokokura. 1998. The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. *J. Dairy Sci*. **81:** 48–53.
22. Modler, H. W., R. C. Mckellar, and N. Yaguchi. 1990. Bifidobacteria and bifidogenic factors. *Can. Inst. Food Sci. Technol. J.* **23:** 29–41.
23. Namba, Y., Y. Hidaka, K. Taki, and T. Morimoto. 1981. Effect of oral administration of lysozyme or digested bacterial cell walls on immunostimulation in guinea pigs. *Infect. Immun*. **31:** 580–583.
24. Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, and S. Calminen. 1999. Probiotics: Mechanisms and established effects. *Int. Dairy J*. **9:** 43–52.
25. Park, S. Y., G. E. Ji, Y. T. Ko, H. K. Jung, Z. Ustunol, and J. J. Pestka. 1999. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of *Bifidobacterium*. *Int. J. Food Microbiol*. **46:** 231–241.
26. Pereyra, B. S., R. Falcoff, E. Falcoff, and D. Lemonnier. 1991. Interferon induction by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in mice. *Eur. Cytokine Netw*. **2:** 299–303.
27. Salminen, S., C. Bouley, M. C. Boutron-Ruault, J. H. Cummings, A. Franck, G. R. Gibson, E. Isolauri, M. C. Moreau, M. Roberfroid, and I. Rowland. 1998. Functional food science and gastrointestinal physiology and function. *Br. J. Nutr*. **80:** S147–S171.
28. Sakagami, H., Y. Kuroiwa, M. Takeda, H. Ota, K. Kazama, T. Naoe, Y. Kawazoe, S. Ichikawa, H. Kondo, T. Yokokura, and M. Shikita. 1992. Distribution of TNF endogenously induced by various immunopotentiators and *Lactobacillus casei* in mice. *In Vivo* **6:** 247–254.
29. Seikine, K., E. W. Seikine, T. Toida, T. Tatsuki, T. Kawashima, and Y. Hashimato. 1994. Induction and activation of tumoricidal cells *in vivo* and *in vitro* by the bacterial cell wall of *Bifidobacterium infantis*. *Bifidobact. Microfl*. **13:** 65–77.
30. Shida, K., K. Makino, A. Morishita, K. Takamizawa, S. Hachimura, A. Ametani, T. Sato, Y. Kumagai, S. Habu, and S. Kaminogawa. 1998. *Lactobacillus casei* inhibits antigen induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. *Int. Arch. Allergy Immunol*. **115:** 278–287.
31. Yoshioka, H., K. Iseki, and K. Fujita. 1983. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* **72:** 317–321.

J. Microbiol. Biotechnol. (2006), 16(4), 584–589

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Modulatory Activity of *Bifidobacterium* sp. BGN4 Cell Fractions on Immune Cells

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Received: August 30, 2005
Accepted: October 24, 2005

Abstract Bifidobacteria has been suggested to exert health promoting effects on the host by maintaining microbial flora and modulating immune functions in the human intestine. We assessed modulatory effects of the different cell fractions of *Bifidobacterium* sp. BGN4 on macrophage cells and other immune cells from the spleen and Peyer's patches (PP) of mouse. Cell free extracts (CFE) of the BGN4 fractions induced well-developed morphological changes in the macrophages and increased the phagocytic activity more effectively than other fractions in the mouse peritoneal cells. Nitric oxide (NO) production was significantly reduced by both the cell walls (CW) and CFE in the cultured cells from the spleen and PP. The production of interleukin-6 (IL-6) and interleukin-10 (IL-10) was eminent in the spleen cells treated with experimental BGN4 cell fractions. However, in the PP cells, IL-6 was slightly decreased by the treatment with the whole cell (WC) and CW, whereas IL-10 was significantly increased by the treatment with the CW and CFE. These results suggest that different types of bifidobacterial cell fractions may have differential immunomodulatory activities depending on their location within the host immune system.

Key words: *Bifidobacterium* sp. BGN4, immune cell, phagocytosis, NO, IL-6, IL-10

Mucosal epithelial cells and microflora cover the large intestines and prevent colonization of the gut by pathogens. Probiotics are strains of microorganisms that confer health benefits to the host. Their beneficial effects include the production of various antimicrobial products, competitive exclusion of enteric pathogens, and modulation of mucosal immune responses [24]. As a probiotic microorganism, *Bifidobacterium* is a nonpathogenic, Gram-positive, and anaerobic bacteria that inhabits the intestinal tracts of humans and animals. *Bifidobacterium* is the most abundant flora in breast-fed babies [1]; however, the proportion of *Bifidobacterium* continuously decreases after the weaning period. The reduction of bifidobacterial flora was also observed in Crohn's disease patients through an epidemiological study [30]. *Bifidobacterium* showed an immunopotentiating activity in the culture of macrophages and lymphocytes [5, 13, 21], in addition to a mitogenic response in the spleen and PP [6, 9, 10, 12].

Macrophages play a critical role in a host's defense system through physical uptake activity and secretion of immune mediators such as NO and various cytokines [16, 28], which inhibits tumor cells, bacteria, fungi, and parasites. Moreover, the macrophages execute a conservative process through engulfment of extracellular materials through phagocytosis [31]. The PP are collections of subepithelial lymphoid follicles burgeoning amongst villi and distributed throughout the intestine, mainly on the antimesentric side. A mixture of lymphocytes, macrophages, and dendritic cells found on top of the follicle towards the gut lumen [8] are involved in the induction of a mucosal immune response in the intestine [17, 18]. The secretion of the various immune mediators by the intestinal immune cells needs to be controlled at an appropriate level since they can potentially injure host cells and tissues [3, 4]. Thus, investigation of the secretion and regulation of immune mediators is warranted, when probiotic strains are considered for the modulation of immune balance.

In our previous study, *Bifidobacterium* sp. BGN4 was found to exhibit a prominent adhesive capacity for intestinal epithelial cells [11] as well as modulatory activity on the production of IL-6 and tumor necrosis factor-α (TNF-α) [15]. To further elucidate the immunomodulatory mechanisms of *Bifidobacterium* sp. BGN4, we examined macrophage activation by morphological differentiation and antigen uptake using a macrophage cell line, RAW 264.7, and observed

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secretions of NO, IL-6, and IL-10 in the spleen and PP cells.

MATERIALS AND METHODS

Experimental Animals

BALB/c and C3H/He mice were purchased from the laboratory animal center of Seoul National University. Eight- to twelve-week-old mice were used in this study.

Bifidobacterium Cultures

The identification and experimental use of *B. bifidum* BGN4 was previously reported [21]. All strains were cultured and subcultured anaerobically in MRS broth (Difco, MI, U.S.A.) containing 5% lactose (w/v, MRSL) at 37°C until the late-log phase. Cells were collected by centrifugation at 1,000 ×*g* for 15 min at 4°C and washed twice with PBS (phosphate-buffered saline) followed by a final washing with distilled water. Freeze-lyophilized BGN4 was resuspended with the cell culture medium to obtain a desired bacterial concentration on a dry weight basis. BGN4 was heat-killed before introduction into the cell culture plate by heating at 95°C for 30 min. Heat-killed cultures were aliquoted and stored at −80°C until use.

Preparation of *Bifidobacterium* sp. BGN4 Cell Fractions

Harvested BGN4 cells were fractionated by a modification of the method according to Okitsu-Negishi *et al.* [20]. Cells grown in the MRS medium were pelleted by centrifugation (1,000 ×*g* for 20 min). The pellets were washed twice with PBS and were centrifuged again. The packed cells were homogeneously suspended in 30 ml of distilled water followed by disintegration by French Press (Spectronic, NY, U.S.A.). The whole cells were removed from the suspension by centrifugation at 2,000 ×*g* for 20 min. The cell walls were sedimented by centrifugation at 15,000 ×*g* for 45 min at 4°C, and the supernatant was used as the CFE. Each fraction was freeze dried (Ilshin, Korea) and resuspended with Dulbecco's modified Eagle's medium (DMEM) (Sigma, MO, U.S.A.) to the desired bacterial concentration on a dry weight basis. Suspended bacterial cells were stored at −20°C until use.

Cell Culture

RAW 264.7 cells were grown to confluence in sterile tissue culture dishes (Costar, MA, U.S.A.) and gently detached by repeated pipetting. Spleens were aseptically extirpated, and single-cell suspensions were prepared by a standard mechanical disruption procedure. The PP was also aseptically detached and washed by stirring at 37°C in RPMI 1640 (containing 1 mg/ml of collagenase IV, Sigma, MO, U.S.A.). After additional washes with RPMI1640 [containing 2% fetal bovine serum (FBS)], the cell suspensions were passed through a 200-mesh nylon membrane to remove incompletely dissociated epithelial tissue sheet, and then washed twice with RPMI 1640 (containing 2% FBS). All experimental cells were cultured in DMEM or RPMI 1640 supplemented with 10% (v/v) FBS (BioWhittaker, MD, U.S.A.) and 1% (v/v) penicillin-streptomycin (Gibco Brl, NY, U.S.A.). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 (Sanyo, Japan) for 48 h. Cell number and viability were assessed by the trypan blue dye exclusion method [27].

Chemicals and Reagents

IL-6, IL-10, purified antibodies to IL-6 or IL-10 (rat anti-mouse), and biotinylated rat anti-mouse IL-6 or IL-10 were obtained from PharMingen (San Diego, CA, U.S.A.). DMEM and FBS were obtained from Gibco Laboratories (Chagrin Falls, IL, U.S.A.). Tetramethylbenzidine (TMB) was purchased from Fluka Chemical Corp (Ronkonkoma, NY, U.S.A.).

Scanning Electron Microscopy

A 2×10^5 cells/ml sample of RAW 264.7 cells was incubated on a coverslip to use bottom-attached cells. Overnight-grown RAW 264.7 cells were co-cultured with BGN4 cell fractions for 24 h. After 24 h of incubation, the cell media and residual particles were decanted, and the cell-attached coverslips were prefixed with glutaraldehyde, washed, and rinsed in a cacodylate buffer. After fixation on osmium tetraoxide, the cells were stained with uranyl acetate and dehydrated in a series of graded ethanols. The cell coverslips were critical-point dried using carbon dioxide, and then coated with gold and observed under the scanning electron microscope (SEM). SEM analysis was accomplished with the help of the National Institute of Crop Science (Suwon, Korea).

Phagocytosis Assay

The cells in peritoneal exudates of the sacrificed BALB/c mice were collected by washing the peritoneal cavity with autoclaved PBS [29]. The cells were diluted to 2×10^5 cells/ml and incubated on coverslips for 2 h with the BGN4 cell fractions. After washing with PBS, 4×10^5 *Candida albicans* cells were co-incubated with peritoneal cells on the coverslip for 45 min. After washing again with PBS, the coverslips were stained with Wright stain for 10 min. Phagocytic activity was expressed as the percentage of phagocytic cells that had phagocytosed *C. albicans*.

Measurement of NO

The concentration of nitrite in the cell culture supernatant was measured using the Griess Reagent System (Promega, Madison, U.S.A.), which is based on the chemical reaction between sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid)

conditions. At first, 50 µl of each experimental sample was mixed with 50 µl of the sulfanilamide solution and incubated for 5 min at room temperature. After incubation, 50 µl of the NED solution was added and incubated for 5 min at room temperature in the dark. Then, the optical density was measured within 30 min in a plate reader with a filter between 520–550 nm.

Quantification of IL-6 and IL-10

The production of IL-6 and IL-10 was detected by ELISA (Enzyme Linked ImmunoSorbent Assay) according to Dong *et al.* [2]. The ELISA plates were read at 450 nm on a V_{max} Kinetic Microplate Reader (Molecular Devices, CA, U.S.A.). IL-6 and IL-10 were quantitated using V_{max} Software (Molecular Devices).

Statistical Analysis

Each set of experiments was performed at least three times. All data were presented as mean±SEM. The data were analyzed by one-way analysis of variance (ANOVA) using the SAS system (SAS Institute Inc., NC, U.S.A.) and t-test. A probability of $p<0.05$ was used as the criterion for statistical significance.

Results and Discussion

Effect of BGN4 Cell Fractions on Macrophage Activation

Previously, our data showed that various fractions of *Bifidobacterium* sp. BGN4 exerted differential effects on the production of cytokines by the macrophage cell line, RAW 264.7 [15]. In this study, we assessed the macrophage activation capacity of BGN4 cell fractions through the SEM and phagocytosis analysis. Macrophage cells stimulated with WC or CFE were larger in size, as depicted by the enlarged surface area [Fig. 1A(b) and (c)] than the control [Fig. 1A(a)]. Furthermore, macrophage cells treated with CFE expressed more differentiated pseudopodia than those treated with WC.

C. albicans is a ubiquitous pathogenic fungus associated with infections of the immunocompromised host [29]. Thus, an efficient and prompt clearance of these organisms is critical in innate immune systems. In this context, we performed a phagocytosis test with the murine peritoneal cells, which consist largely of phagocytic immune cells. Peritoneal cells treated with WC, CW, and CFE showed significantly increased levels of phagocytosis, with CFE showing higher stimulatory activity than WC or CW (Fig. 1B).

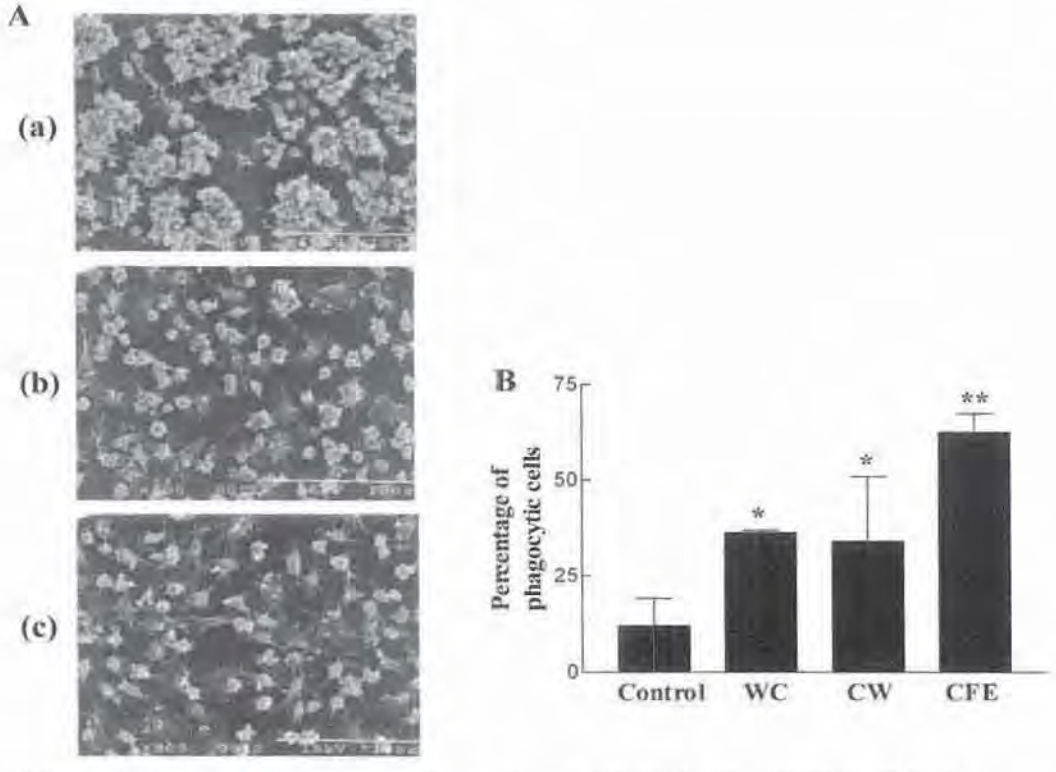

Fig. 1. Effect of BGN4 cell fractions on the activation of macrophage. RAW 264.7 cells and BGN4 cell fractions were co-cultured for 24 h and observed using a scanning electron microscope.

A. (a) Control (RAW 264.7 cells), (b) BGN4 WC (50 µg/ml) for 24 h, (c) BGN4 CFE (50 µg/ml) for 24 h. *C. albicans* (4×10^5 cells) was co-incubated with mouse (BALB/c) peritoneal cells and pretreated with BGN4 cell fractions on the coverslip for 45 min. Phagocytic activity was expressed by the percentage of phagocytic cells **B**. The results are expressed as mean±SEM of triplicates (* is defined different from the control, ** is defined by increased activity than the same concentration of WC or CW, $p<0.05$).

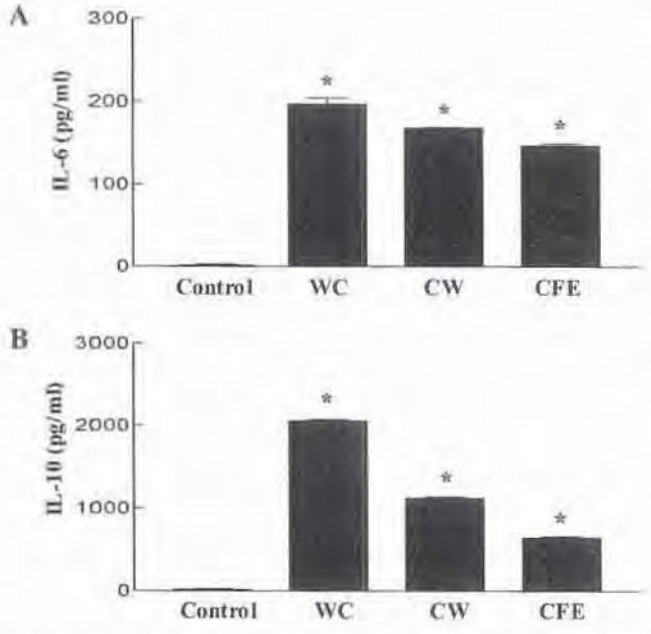

Fig. 2. Effect of BGN4 cell fractions on the production of NO by splenocytes and PP.
Splenocytes (**A**) and PP cells (**B**) (5×10^5 cell/ml) were cultured for 48 h in the presence of BGN4 cell fractions for the detection of NO. The BGN4 cell fractions were preheated at 95°C for 30 min. Data are presented as mean±SEM of triplicates (* is defined different from control, $p<0.05$).

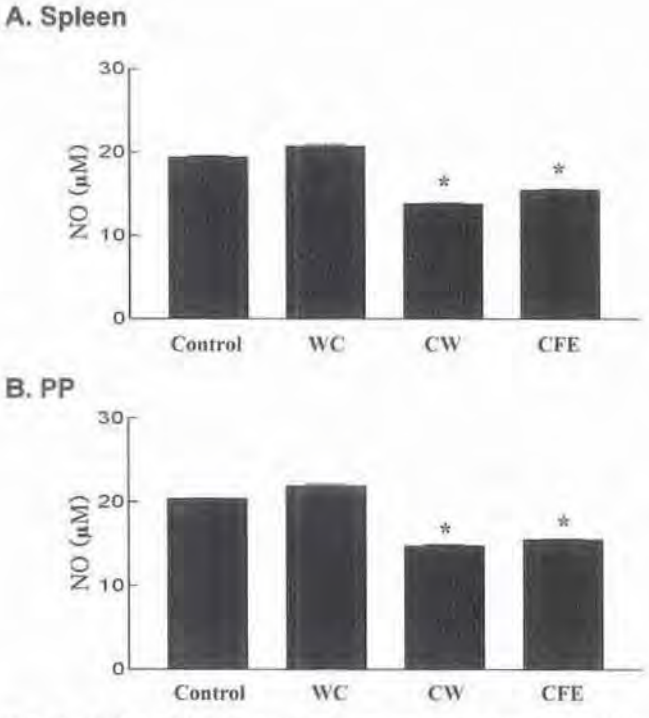

Fig. 3. Effect of BGN4 cell fractions on the production of IL-6 and IL-10 in the splenocytes culture.
Splenocytes (5×10^5 cells/ml) were cultured for 48 h in the presence of various bacterial fractions for the detection of IL-6 (**A**) and IL-10 (**B**) production. The bacterial fractions were preheated at 95°C for 30 min. Data are presented as mean±SEM of triplicates (* is defined as significantly different from the control, $p<0.05$).

Inhibitory and Regulatory Effect of BGN4 Cell Fractions on NO, IL-6, and IL-10

Figures 2A and 2B indicate that WC did not show any significant difference in the production of NO, when compared with the control. Interestingly, CW and CFE showed significantly reduced levels of NO production. NO is mainly produced from macrophage and monocytes, and is an important mediator of macrophage phagocytosis [12]. However, NO-mediated cell damage enhances the release of a proinflammatory mediator from the macrophages. Enhancement of IL-8 and TNF-α release is also partially NO-dependent in the activated peritoneal neutrophils [24]. Our results suggest that the immunomodulatory effects of *Bifidobacterium* are exerted in a rather sophisticated manner in the spleen and PP than mere reflection of the stimulatory effects observed in the macrophage cells, possibly causing a noted differential effect of WC compared with CW and CFE.

Probiotics are suggested to have direct effects on the intestinal lumen or intestinal immune cells via cytokine induction [19]. Macrophage and lymphocytes play a central role in cell-mediated and humoral immunity through the release of different cytokines such as TNF-α, IL-6, and IL-10 [32]. In the spleen cells, strong IL-6 and IL-10 productions by each fraction were observed, with WC showing the highest secretion level of cytokines (Fig. 3). In PP, the patterns of the production of IL-6 and IL-10 were considerably different from those in the spleen cells. Interestingly, WC and CW significantly decreased IL-6 production, although by a slight margin. In the case of IL-10 production, CW and CFE showed significantly increased levels, compared with the control (Fig. 4). The results from the PP might reflect better the *in vivo* situation than those from the spleen cells, since the PP continuously contacts with the luminal intestinal bacteria (Figs. 3 and 4) [25]. Furthermore, the differences in the cell composition between spleen and PP could have been due to the differential effects of the different BGN4 cell fractions. Additionally, it was of interest to note that CFE had stronger stimulatory activity for IL-6 and IL-10 production in the PP cells, whereas WC showed greater stimulation in the spleen cells (Figs. 3 and 4).

Although BGN4 cell fractions apparently activated the immune cells, the stimulatory effects of BGN4 varied, depending on the types of the BGN4 cell fractions or the host immune cells. Taken together, BGN4 induced cell differentiation and phagocytosis, and promoted secretion of the anti-inflammatory cytokine, IL-10. Commensal microorganisms continuously interact with the epithelial cell layers and present a number of innate immunity associated antigens via receptors for the pathogen-associated molecular patterns (PAMS). A recent report showed that nonpathogenic

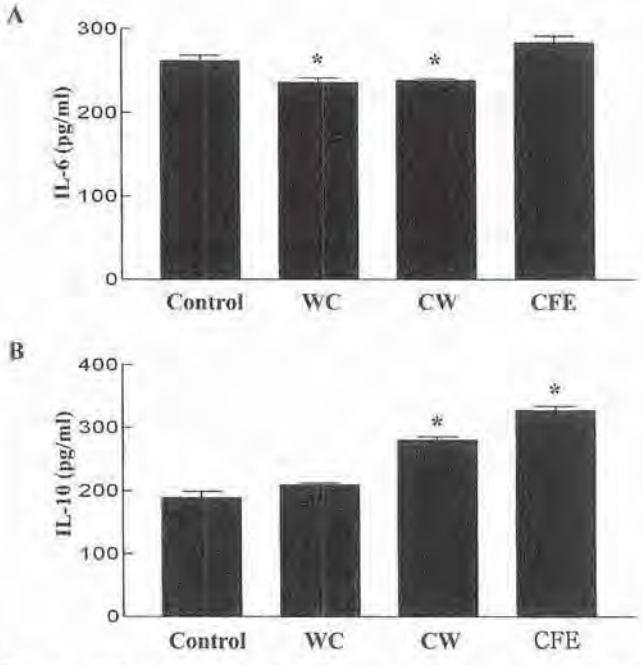

Fig. 4. Effect of BGN4 cell fractions on the production of IL-6 and IL-10 in the PP.

PP cells (5×10^5 cells/ml) were cultured for 48 h in the presence of various bacterial fractions for the detection of IL-6 (**A**) and IL-10 (**B**) production. The bacterial fractions were preheated at 95°C for 30 min. The results are expressed as mean±SEM of triplicates (* is defined as significantly different from the control, $p<0.05$).

intestinal bacteria could induce dendritic cells (DC) to migrate into the epithelial layer for antigen sampling from the gut lumen [22]. Particularly in the gut, recruited DC by luminal antigen, including intestinal bacteria, is important for the induction and maintenance of peripheral self-tolerance [7]. Further study on the strain-dependent or bacterial fraction-dependent effects on cytokine production and immune cell activation may deepen the understanding of the role of *Bifidobacterium* in the intestinal immune system.

Acknowledgment

This work was supported by a grant from the Korean Ministry of Science and Technology of Korea (Grant no. M1-0302-00-0098).

References

1. Bezkorovany, A. 1989. Ecology of bifidobacteria, pp. 29–72. *In* Bezkorovainy, A. and R. Miller-Catchpole. (eds.), *Biochemistry and Physiology of Bifidobacteria.* CRC press, Florida, U.S.A.
2. Dong, W., J. I. Azcona-Olivera, K. H. Brooks, J. E. Linz, and J. J. Pestka. 1994. Elevated gene expression and production of interleukins 2, 4, 5 and 6 during exposure to vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. *Toxicol. Appl. Pharmacol.* **127:** 282–290.
3. Fukuo, K., T. Inoue, S. Morimoto, T. Nakahashi, O. Yasuda, S. Kitano, R. Sasada, and T. Ogihara. 1995. Nitric oxide mediates cytotoxicity and basic fibroblast growth factor release in cultured vascular smooth muscle cells. A possible mechanism of neo vascularization in atherosclerotic plaques. *J. Clin. Invest.* **95:** 669–676.
4. Fuseler, J. M., E. M. Conner, J. M. Davis, R. E. Wolf, and M. B. Grisham. 1997. Cytokine and nitric oxide production in the acute phase of bacterial cell wall-induced arthritis. *Inflammation* **21:** 113–131.
5. Hatcher, G. E. and R. S. Lambrecht. 1993. Augmentation of macrophage phagocytic activity by cell free extracts of selected lactic acid-producing bacteria. *J. Dairy Sci.* **76:** 2485–2492.
6. Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S. Kaminogawa. 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M 101-4. *Biosci. Biotech. Biochem.* **61:** 312–316.
7. Huang, F. P., N. Platt, M. Wykes, J. R. Major, T. J. Powell, C. D. Jenkins, and G. G. MacPherson. 2000. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J. Exp. Med.* **191:** 435–443.
8. Hussain, N., V. Jaitley, and A. T. Florence. 2001. Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics. *Adv. Drug Deliv. Rev.* **50:** 107–142.
9. Kado-Oka, Y., S. Fujiwara, and T. Hirota. 1991. Effects of bifidobacteria cells on mitogenic response of splenocytes and several functions of phagocytes. *Milchwissenshaft* **46:** 626–630.
10. Kim, H.Y., J. H. Yang, and G. E. Ji. 2005. Effect of bifidobacteria on production of allergy-related cytokines from mouse spleen cells. *J. Microbiol. Biotechnol.* **15:** 265–268.
11. Kim, I. H., M. S. Park, and G. E. Ji. 2003. Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 cells. *J. Microbiol. Biotechnol.* **13:** 276–281.
12. Kim, Y. M., T. R. Billiar, and J. R. Lancaster Jr. 1996. Reactive oxygen and nitrogen metabolites and related enzymes, pp. 171.1–171.10. *In* L. A. Hezenberg, D. M. Weir, L. A. Herzenberg, and C. Blackwell (eds.), *Weir's Handbook of Experimental Immunology*, **Vol. 4**. *The Integrated Immune system*. Blackwell Science, Cambridge, U.S.A.
13. Lee B. H. and G. E. Ji. 2005. Effect of *Bifidobacterium* cell fractions on IL-6 production in RAW 264.7 macrophage cells. *J. Microbiol. Biotechnol.* **15:** 740–744.
14. Lee, J., A. Ametani, A. Enomoto, Y. Sato, H. Motoshima, F. Ike, and S. Kaminogawa. 1993. Screening for the immunopotentiating activity of food microorganisms and enhancement of the immune response by *Bifidobacterium adolescentis* M101-4. *Biosci. Biotech. Biochem.* **57:** 2127–2132.
15. Lee, M. J., Z. Zang, E. Y. Choi, H. K. Shin, and G. E. Ji. 2002. Cytoskeleton reorganization and cytokine production

of macrophages by bifidobacterial cells and cell-free extracts. *J. Microbiol. Biotechnol.* **12:** 398–405.
16. Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Synder, and S. W. Russell. 1993. Expression of the nitric oxide synthase gene in mouse macrophage activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. *J. Biol. Chem.* **268:** 1908–1913.
17. Makala, L. H. C., T. Kamada, Y. Nagasawa, I. Igarashi, K. Fujisaki, N. Suzuki, T. Mikami, K. Haverson, M. Bailey, C. Stokes, and P. Bland. 2001. Ontogeny of pig discrete Peyer's patches: Expression of surface antigens. *J. Vet. Med. Sci.* **63:** 625–636.
18. Makala, L. H. C., Y. Nishikawa, T. Kamada, H. Suzuki, X. Xuan, I. Igarashi, and H. Nagasawa. 2001. Comparison of the accessory activity of murine peritoneal cavity macrophage derived dendritic cells and peritoneal cavity macrophage in a mixed lymphocyte reaction. *J. Vet. Med. Sci.* **63:** 1271–1277.
19. Marteau, P., P. Seksik, P. Lepage, and J. Dore. 2004. Cellular and physiological effects of probiotics and prebiotics. *Mini Rev. Med. Chem.* **4:** 889–896.
20. Okitsu-Negishi, S., I. Nakano, K. Suzuki, S. Hashira, T. Abe, and K. Yoshino. 1996. The induction of cardioangitis by *Lactobacillus casei* cell wall in mice. I. The cytokine production from murine macrophages by *Lactobacillus casei* cell wall extract. *Clin. Immunol. Immunopathol.* **78:** 30–40.
21. Park, S. Y., G. E. Ji, Y. T. Ko, H. K. Jung, Z. Ustunol, and J. J. Pestka. 1999. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commensal isolates of *Bifidobacterium*. *Int. J. Food Microbiol.* **46:** 231–241.
22. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2:** 361–367.
23. Sekine, K., T. Kasashima, and Y. Hashimoto. 1994. Comparison of the TNF-α levels induced by human-derived *Bifidobacterium longum* and rat-derived *Bifidobacterium animalis* in mouse peritoneal cells. *Bifidobact. Microfl.* **13:** 79–89.
24. Shortt, C. 1999. The probiotic century: Historical and current perspectives. *Trends Food Sci. Tech.* **10:** 411–417.
25. Smith, D. W. and C. Nagler-Anderson. 2005. Preventing intolerance: The induction of nonresponsiveness to dietary and microbial antigens in the intestinal mucosa. *J. Immunol.* **174:** 3851–3857.
26. Southey, A., S. Tanaka, T. Murakami, H. Miyoshi, T. Ishizuka, M. Sugiura, K. Kawashima, and T. Sugita. 1997. Pathophysiological role of nitric oxide in rat experimental colitis. *Int. J. Immunopharmacol.* **19:** 669–676.
27. Strober, W. 1991. Trypan blue test for cell viability, pp. A.3.3–A3.4. *In* Coligan, J. E., A. M. Kruisbeek, D. H. Marguiles, E. M. Shevach, and W. Strober (eds.), *Current Protocols in Immunology*. Greene Pub. Associates and Wiley-Interscience, New York, U.S.A.
28. Synder, S. H. and D. S. Bredt. 1992. Biological roles of nitric oxide. *Sci. Am.* **266:** 68–77.
29. Szabo, I., L. Guan, and T. J. Rogers. 1995. Modulation of macrophage phagocytic activity by cell wall components of *Candida albicans*. *Cell. Immunol.* **164:** 182–188.
30. Teitelbaum, J. E. and W. A. Walker. 2002. Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. *Annu. Rev. Nutr.* **22:** 107–138.
31. Underhill, D. M. and A. Ozinsky. 2002. Phagocytosis of microbes: Complexity in action. *Annu. Rev. Immunol.* **20:** 825–852.
32. Vinderola, C. G., M. Medici, and G. Perdigón. 2004. Relationship between interaction sites in the gut, hydrophobicity, mucosal immunomodulating capacities and cell wall protein profiles in indigenous and exogenous bacteria. *J. Appl. Microbiol.* **96:** 230–243.

Clinical Immunology (2007) **123**, 30—39

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Oral feeding of *Bifidobacterium bifidum* (BGN4) prevents CD4$^+$ CD45RB^{high} T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation

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Received 9 March 2006; accepted with revision 8 November 2006
Available online 10 January 2007

KEYWORDS
IBD;
Probiotics;
Bifidobacterium bifidum BGN4;
Inflammation;
Th1

Abstract Probiotics have been considered as preventive agents for the control of inflammatory bowel disease (IBD). In this study, we assessed the immunomodulatory effect of *Bifidobacterium bifidum* BGN4 on the control of IBD using the CD4$^+$ CD45RB^{high} T cell transfer disease model. The mice were fed for 4 weeks with either a conventional diet containing only skim milk or a diet containing skim milk with 0.3% (w/w) BGN4. The BGN4-fed mice showed normal weight growth, fewer clinical symptoms such as thickened wall and inflammatory cell infiltration, and lower levels of CD4$^+$ T lymphocyte infiltration and inflammatory cytokine productions than the skim milk-fed mice with IBD in the large intestine. Suppression of these cytokine productions, particularly IFN-γ and MCP-1, through BGN4 treatment was also observed in the *in vitro* co-culture between intestinal epithelial cells and T cells. These findings suggested that a BGN4 supplemented diet could be helpful for the control of aberrant immune responses in the intestinal tissue.

Introduction

Inflammatory bowel disease (IBD) has been divided pathologically into two categories of morbus, Crohn's disease (CD) and ulcerative colitis (UC), but the mechanism involved in the immunopathological and genetic basis of IBD is not yet fully understood [1]. Experimental and epidemiological studies suggest that the host disorders in the function of

Abbreviations: IBD, inflammatory bowel disease; SCID, severe combined immunodeficiency; Th, T helper; Ig, immunoglobulin; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor-α; MRS broth, de Man, Rogosa, Sharpe broth; PBS, phosphate buffered saline; mAb, monoclonal antibody; CD, Crohn's disease; UC, ulcerative colitis; TLR, toll-like receptor; DC, dendritic cell; KO, knock out; GITR, glucocorticoid-induced TNFR family-related gene.

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doi:10.1016/j.clim.2006.11.005

the immune system are the major contributing factors for the development of IBD [1]. For example, disruption or manipulation of genes encoding cytokines, cytokine receptors, or immunological molecules (e.g. T cell receptor α chain) results in the induction of IBD symptoms, which are mainly observed in the large intestinal compartments of experimental mice [2,3]. Furthermore, the microenvironment of the intestinal tract has been shown to contribute to the development of IBD. Thus, it was demonstrated that gene manipulated mice lacked IBD development in germ-free conditions [4]. It was also shown that antibiotic treatment attenuated the disease sensitivity of the gene manipulated mice [4–6]. These findings imply that the onset of IBD is associated with the interaction of the host immune system and the bacterial flora residing in the gut of the host.

Recent findings have shown the beneficial effects of probiotics on IBD [7]. Probiotics are strains of defined microorganisms that confer health benefits through many diverse ways. These include the production of various antimicrobial products, competitive exclusion of enteric pathogens, and modulation of mucosal immune responses [8]. Based on the anti-inflammatory effect of probiotics *in vivo* and *ex vivo* [7,9], several strains have been examined for their potential use in IBD therapy [10,11]. In particular, probiotic application to prophylactic supplements has been investigated [8]. Moreover, genetically modified probiotics system has been also applied to murine colitis models using artificially engineered *Lactococcus lactis* secreting inhibitory cytokines such as interleukin (IL)-10 [12].

Bifidobacterium is the most abundant flora in breast-fed babies [13], however the amount of flora sequentially decreases after the weaning period. The reduction of bifidobacterial flora is also observed in Crohn's disease patients through epidemiological studies [14]. Supplemental *Bifidobacterium* has been shown to execute health promoting effects by their immunomodulatory function and has been tested for their application in the possible prevention and therapy of IBD [10,15]. Bifidobacteria secretes non-protein, lipophilic antibacterial compounds and inhibits colonization of diarrheagenic bacteria [15]. Significantly reduced levels of inflammatory cytokines such as TNF-α and IFN-γ are observed in the culture of intestinal immunocytes from *Bifidobacterium infantis*-fed mice [10]. Thus, *Bifidobacterium* is considered to play an important role for the creation of a well balanced intestinal immunity.

In our previous study, *Bifidobacterium bifidum* BGN4 (BGN4), when compared with the other strains, exhibited a prominent adhesive capacity for intestinal epithelial cells, which is one of the desirable properties for a probiotical effect [16]. BGN4 also exhibited high immunomodulatory activities such as IL-10 production when the BGN4 was co-treated with a pathogenic bacterial fraction (unpublished data). These findings allow for the hypothesis that the oral feeding of BGN4 is effective for the prevention of IBD due to its anti-inflammatory properties. To address this hypothesis, we employed the $CD4^+$ $CD45RB^{high}$ T cell transfer model for the induction of IBD because the disease is well established as the Th1-type cytokine-derived hyper-response associated disease [1,17–19]. In addition, it has been shown that the symptoms were spontaneously and specifically developed in the colon [18,19] as the diversity of enteric flora decreased in the diseased mice [20]. These results allowed us to consider the critical role of probiotic BGN4 for the control of IBD. Thus, the aim of this study is to investigate the preventive effect of BGN4 on the $CD4^+$ $CD45RB^{high}$ T cell-mediated IBD.

Materials and methods

Experimental animal

Eight-week-old female BALB/c ($H\text{-}2^d$), C57BL/6 ($H\text{-}2^b$), C.B-17/Icr ($H\text{-}2^d$), and severely combined immunodeficient (SCID) C.B-17/Icr (C.B-17/Icr-Scid/Scid; $H\text{-}2^d$) mice were purchased from Japan Clea (Tokyo, Japan). All mice were housed in microisolator cages and fed sterilized food and tap water *ad libitum*.

Bifidobacterial cultures and diet

The identification and characterization of BGN4 have been previously reported [21]. BGN4 were cultured anaerobically in MRS broth (Difco, Detroit, MI, USA) containing 5% lactose (wt/vol, MRSL) at 37 °C until late log phase. The cells were collected by centrifugation at 1000×*g* for 15 min at 4 °C and washed twice with PBS followed by a final washing with distilled water. They were dried by Speed-Vac (Speed-Vac Instruments, INC., NY, USA). Lastly, freeze-lyophilized BGN4 (10^{11} CFU/g) was mixed with skim milk, which was used as a cryoprotectant agent and mixed in the regular diet. Thus, the diet containing 0.3% (per gram of regular diet) skim milk with BGN4 (BGN4-fed group) and the diet containing only 0.3% skim milk (skim milk-fed group) were prepared and used in this study.

Murine experimental design

The $CD4^+$ $CD45RB^{high}$ T cell transfer colitis model was used [17–19]. Purified splenic $CD4^+$ $CD45RB^{high}$ T cells (5×10^5 cells) were adoptively transferred by intraperitoneal injection into the recipient C.B-17/Icr SCID mice. Immediate after the transfer of $CD4^+$ $CD45RB^{high}$ T cells to the recipient, the BGN4-fed group consumed 0.3% probiotics powder of BGN4 in skim milk/g of regular diet, while the control group consumed the diet containing 0.3% skim milk (per gram of regular diet) without BGN4. Each group of mouse consumed 3–5 g of diet per day, mice in the BGN4-fed group therefore consumed $0.9–1.5\times10^9$ organisms per day. Before sacrificing of the mice, a fecal sample was collected and analyzed for bacteria at the Research Foundation for Microbial Diseases of Osaka University (Osaka, Japan) [22]. The mice were individually monitored for their weight loss, loose stools, bloody diarrhea, and rectal prolapse [23]. Mice from both groups were sacrificed and analyzed after 4 weeks from the adoptive transfer.

Isolation of mononuclear cells from the spleen, small intestine, and large intestine

The spleen was aseptically extirpated, and single-cell suspensions were prepared by a standard mechanical disruption procedure. Intestinal lymphocytes were isolated from

the lamina propria region of the intestine as previously described [24]. Briefly, the small and large intestines were dissected after removing the Peyer's patch. Freshly dissected intestine was opened longitudinally, washed thoroughly, and cut into small fragments. Epithelial cells and intraepithelial lymphocytes (IEL) were removed from the intestinal tissue by incubating in RPMI1640 (GIBCO) (containing 1 mM EDTA) and shaking vigorously with RPMI1640 (containing 2% FCS). The specimens were then minced and stirred at 37 °C in RPMI1640 (containing 1 mg/ml of collagenase IV, Sigma, St. Louis, MO, USA). After additional washes with RPMI1640 (containing 2% FCS), cell suspensions were passed through a 40-μm membrane to remove incompletely dissociated epithelial tissue sheets and were then separated using discontinuous 40% and 75% percoll (Amersham Pharmacia Biotech, Amersham) gradient centrifugation. After washing twice with RPMI1640 (containing 2% FCS), the cells were used for the cell culture and flow cytometric analysis.

FACS analysis and cell sorting

Isolated mononuclear cells from the spleen and intestine were pre-incubated with anti-CD16/32 mAb (2.4G2; BD Pharmingen) for 15 min at room temperature for pre-occupation of non-specific Fc binding site [23]. The following Abs were purchased from BD Pharmingen and used for staining: FITC- or PE-conjugated anti-mouse CD4 (RM4-5), CD69 (H1.2F3) and CD45RB (16A) mAbs. The percentage of cells expressing surface markers and the intensity of expression were determined by FACS Calibur (Becton Dickinson, San Jose, CA, USA) and CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA) [24].

To obtain $CD4^+$ $CD45RB^{high}$ cells, the MACS microbead system (Miltenyi Biotech) was used to deplete the $CD8^+$, $CD11b^+$, $CD11c^+$, $CD19^+$, and $B220^+$ cells by negative selection according to the manufacturer's instruction. The resulting $CD4^+$-enriched population was then labeled with FITC-conjugated anti-CD4 Ab and PE-conjugated anti-CD45RB Ab. Subpopulations of $CD4^+$ cells were isolated by two-color sorting on a FACS Aria (BD Biosciences) [24]. The $CD45RB^{high}$ were defined as the brightest staining cells, which consisted of 40–50% $CD4^+$ T cells. All populations were >98% pure based on the re-analysis by flow cytometer.

Cell culture and cytokine measurement

Isolated mononuclear cells from the spleen (5×10^5 cells/well) and lamina propria of the large intestine (3×10^5 cells/well) were cultured in RPMI1640 containing 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME and 1% penicillin/streptomycin [22]. Culture supernatants were harvested after 48 h and TNF-α, IFN-γ, MCP-1, IL-6, IL-2, IL-4, IL-5, and IL-10 production were determined by a commercially available CBA-kit (BD Biosciences).

Co-culture of CMT93 murine epithelial cell line and lymphocytes together with BGN4

Modified co-culture system was adopted from a previous report [25]. CMT93, derived from a mouse rectal carcinoma ($H\text{-}2^b$), was co-cultured with homologous haplotype lymphocytes from C57BL/6 ($H\text{-}2^b$) to mimic the intestinal environment. For the transwell culture, 6×10^5 cells of CMT93 were seeded into a microporous membrane insert (0.6 cm^2 surface, 0.4 μm pore diameter; Millipore) in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1% penicillin/streptomycin. After confirming CMT93 confluent layer using transepithelial electrical resistance (TEER; Millicell ERS Ohmmeter, Millipore, Eschborn, Germany), the inserts were placed into 24-well plates (Costar, Corning), precoated with 2 μg/ml anti-CD3 mAb (145-2C11; BD Pharmingen), and 2 μg/ml soluble anti-CD28 mAb (37.51, BD Pharmingen) in order to culture with the isolated lymphocytes from the spleen of C57BL/6. The confluence of the cells was confirmed by the TEER exceeding the cut-off point of 480 Ω cm^2. BGN4 (heat killed suspension in DMEM, 100 μg/ml) was added in the upper well and cytokine secretion level of bottom well containing lymphocytes was measured after 48 h of incubation.

Histological analysis

The spleen, small intestine, and large intestine were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) for the analysis of the overall shape, architectural distortion, and the degree of lymphocyte accumulation [24].

Statistical analysis

Significant differences between the mean values were determined by a two-sided Student's *t* test. Data are expressed in a standard error of the mean (SEM). *p* values less than 0.05 are considered statistically significant.

Results

BGN4 rescued body weight loss and tissue hypertrophy in the $CD4^+$ $CD45RB^{high}$ T cell induced IBD

To assess the role of BGN4 feeding on the development of IBD, we employed the murine model of $CD4^+$ $CD45RB^{high}$ T cells transfer to SCID mice [17–19]. Two groups of mice were fed for 4 weeks, one group with a conventional diet containing skim milk and 0.3% (w/w) BGN4 (BGN4-fed group) and a second group with a conventional diet containing skim milk only (skim milk-fed group). We initially measured the body weight loss as one of the well documented monitoring items for IBD symptoms. Consistent with previous studies [17–19], the skim milk-fed mice showed a gradual body weight loss (Fig. 1A). Additionally, we of course observed a normal weight growth in control mice that received whole $CD4^+$ T cells even when mice were fed with skim milk diet. The body weight loss in the skim milk-fed mice was concurrent with other IBD symptoms, such as gross hair, rectal bleeding, and incomplete formation of bowel contents (data not shown). Conversely, the development of body weight loss and other clinical symptoms was not observed in the BGN4-fed group. Further pathological analyses showed that the large intestine of the skim milk-fed mice possessed loose stools and gross colitis, whereas the

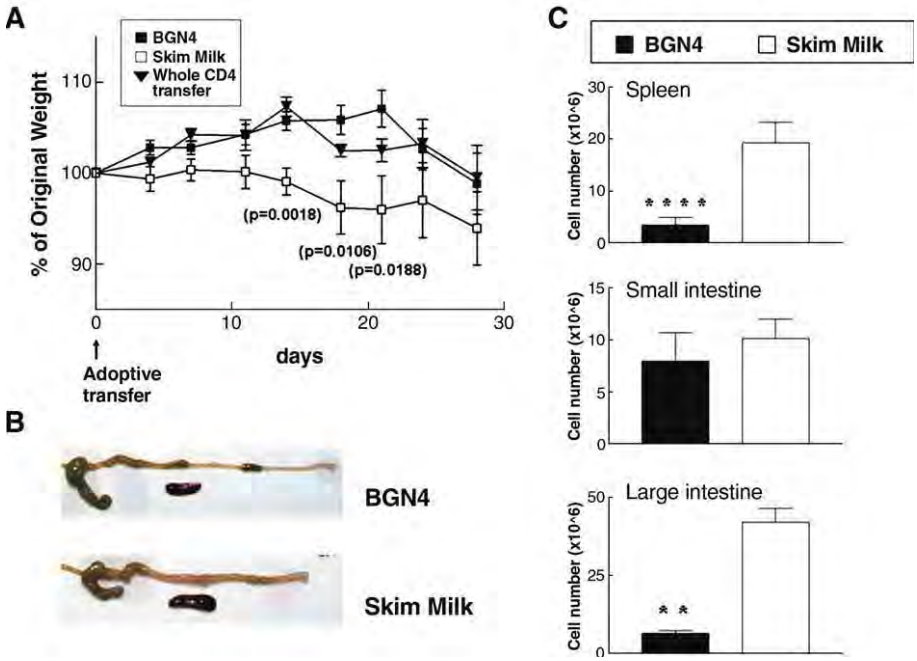

Figure 1 Body weight growth and gross view of the intestine and spleen in BGN4- and skim milk-fed mice. (A) The change in body weight was monitored during 4 weeks in $CD4^+$ $CD45RB^{high}$ transferred SCID mice treated with BGN4 or with skim milk. As a control, a group of mice received whole $CD4^+$ T cells was fed with skim milk. Each group contained 5 mice. Data are expressed as a mean of standard error (SEM). *P* value means the significant difference between BGN4-fed mice and skim milk-fed mice. (B) After 4 weeks, each group of mice was sacrificed and the condition of the intestine and spleen was examined. (C) Numbers of isolated lymphocytes from the spleen, small intestine, and large intestine were compared between the BGN4-fed mice and the skim milk-fed mice. Data are expressed as the mean value of SEM (**$p = 0.007$, ****$p < 0.0001$ versus skim milk-fed mice).

BGN4-fed mice showed normal shapes of intestinal gross architecture (Fig. 1B). In addition, the total number of isolated lymphocytes in the spleen and large intestine of the skim milk-fed mice was significantly higher than that of the BGN4-fed mice (Fig. 1C). These results demonstrate the preventive effect of BGN4 on the development of IBD and suggest that the administration of probiotic bacteria can contribute to the normalization of homeostasis of the intestinal immunity.

In the next experiment, we analyzed the composition of bacteria following BGN4 administration. The feces were isolated for the analysis of bacterial composition including those of *E. coli* and *Bacteroides*. Interestingly, the number of representative intestinal bacteria, *E. coli* and *Bacteroides*, were focused and did not show any significant modification between the skim milk-fed and the BGN4-fed groups (data not shown). The other bacterial strains such as aerobic Gram-positive rod, anaerobic Gram-negative rod, *Proteus mirabilis*, α-*Streptococcus*, Coagulase-negative staphylococci, and *Enterococcus* species appeared in the fecal analysis, however, significant difference was not observed between the BGN4 and skim milk-fed mice.

Prevention of lymphocyte accumulation in the spleen and large intestine by BGN4 feeding

Since BGN4 effectively prevents IBD symptoms without change of intestinal microflora (Fig. 1), we theorized that BGN4 might modulate the intestinal immune system. To test this hypothesis, histological analysis was performed to examine the infiltration of the lymphocytes into the spleen and large intestine of SCID mice after adoptive transfer. It is well established that a normal spleen is largely composed of white and red pulp. White pulp is circular in structure and is made up of a B cell zone and peripheral sheaths mainly containing T cells, while the red pulp surrounds the white pulp and contains a large number of erythrocytes, macrophage, and DC [26]. In this regard, intact SCID mice showed an immature shape of the spleen, that is, the segmentation of white and red pulps was not clearly identified (Fig. 2A). In the case of the BGN4-fed group, the whole morphology of the spleen looked similar to the pattern of normal ICR mice, although the lymphocytic density was low. In contrast, skim milk-fed mice showed an irregular and highly concentrated lymphocyte accumulation. Moreover, a distinct histological change was seen in the spleen of mice adoptively transferred with $CD4^+$ $CD45RB^{high}$ T cells in this study (Fig. 2A).

H&E staining showed severe lymphocyte accumulation in the lamina propria region of the distal colon in the skim milk-fed group, while a lower level of infiltrated lymphocytes was detected in the BGN4-fed group (Fig. 2B). Interestingly, thickened wall and crypt elongation observed in the large intestine of the skim milk-fed mice were not seen in the BGN4-fed group (Fig. 2B). Moreover, the reduction of goblet cells was ameliorated by the BGN4 feeding (Fig. 2B). Based on the clinical criteria and

Life-style Related Diseases

Yoshikawa T (ed): Food Factors for Health Promotion.
Forum Nutr. Basel, Karger, 2009, vol 61, pp 117–128

Probiotics in Primary Prevention of Atopic Dermatitis

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Abstract

The incidence of allergic diseases has been increasing in industrialized countries during recent years. Although several environmental factors are thought be involved, lack of moderate level of microbial challenges during the infantile period is known to skew the immune status toward the development of allergic diseases. Various strains of probiotics such as *Bifidobacterium*, *Lactobacillus*, and *Lactococcus* have been assessed for their ability to suppress the occurrence of atopic dermatitis (AD) in animal models and human studies. Although the effect of probiotics on allergic responses is different depending on the strains, doses, and experimental protocols, animal studies generally have shown immunomodulatory activities of probiotics including suppression of specific or nonspecific IgE production, reduction of infiltrated eosinophils and degranulated mast cells, potentiation of regulatory T cell cytokines such as IL-10 and TGF-β relative to IL-4 and IL-5, and potentiation of Th1/Th2 activity along with reduced symptoms of AD. Several well-designed double-blind placebo-controlled human studies showed that some probiotic strains administered during perinatal period prevented the occurrence of AD but could not consistently show a reduction in specific or nonspecific IgE or a change in specific immunomodulatory cytokines. Taken together, published results suggest that the administration of selected strains of probiotics during the perinatal period may be helpful in the prevention of AD.

The gastrointestinal (GI) tract is an immune organ which is continuously exposed to antigens in the form of food, normal bacteria, and pathogens. Despite the numerous antigenic challenges, the mucosal immune system ordinarily maintains GI homeostasis through the orchestrated actions of the various mucosal immune cells. *Bifidobacterium* and *Lactobacillus* are major components of the commensal microbes in the GI tract and are frequently used as probiotics. They are known to benefit various physiological responses of the host including immunomodulatory activity. Recently, the potential use of probiotics in the prevention of allergy has drawn considerable attention.

New types of disease are emerging in the modern society. Among those diseases, atopic dermatitis (AD) is one of the most disturbing problems in the developed countries including USA, Europe, and Japan [1]. Prevalence of allergy tends to be lower in the families with a higher number of siblings, and the incidence of allergic diseases is higher in urban areas than rural areas. These observations underline the importance of environmental factors in the occurrence of allergic diseases [1]. In accordance with the phenomenon mentioned above, the so-called 'hygiene hypothesis' was put forward [2]. According to the hygiene hypothesis, the use of vaccine and antibiotics and the improved sanitation reduced the incidence of infectious diseases in children. As a result, immune challenges were reduced, which suppressed the potentiation of Th1- relative to Th2-immunity and increased the occurrence of allergy-related disease in children. However, the development of allergy turned out to be more complicated than a simple Th1/Th2 balance theory since an abnormally high level of Th1 or weak activity of regulatory T cells could lead to the occurrence of allergy or allergy-mediated inflammation. The analysis of epidemiological studies and human clinical studies by Flohr et al. [3] showed that the incidence of hepatitis virus, *Helicobacter pylori*, tuberculosis, and herpes simplex infections in infants and children was associated with higher frequencies of allergic diseases even though these infections are associated with the increase in Th1 immunity at the infected sites. These contradictory circumstances with the hygiene hypothesis and alleged increase in allergy from either too strong Th1- or Th2-mediated immunity called for a new hypothesis that consider regulatory cells T cells such as Treg as a crucial modulator in the prevention of allergic disorders (fig. 1). Mice raised in a germ-free environment failed to develop oral tolerance and had a persistent Th2-dependent immune response, while reconstitution of intestinal microbes during the neonatal period could reverse this immune deviation [4]. Therefore, establishment of normal intestinal microflora may be crucial in the maintenance of normal gut barrier function and development of tolerogenic immune status [5]. In addition, exposure to farm animals, pets, and day care environment during infantile period are known to be helpful for the introduction of benign challenges including various nonpathogenic microorganisms, which leads to the establishment of protective immunity against allergic disorders in infants. Infants with AD or other allergic diseases show less intestinal colonization of *Lactobacillus* or *Bifidobacterium* and more colonization of *Clostridium* relative to nonallergic infants [6]. In this context, the potential immune regulatory effect of the probiotics in regard to the prevention of allergy has attracted considerable interest among clinical doctors, food microbiologists, and nutrition scientists.

Probiotics in Primary Prevention of Atopic Dermatitis in Human Studies

In the study by Alm et al. [7], the incidence of allergy was lower among the children grown in the family frequently eating traditional lactic bacteria-fermented foods than

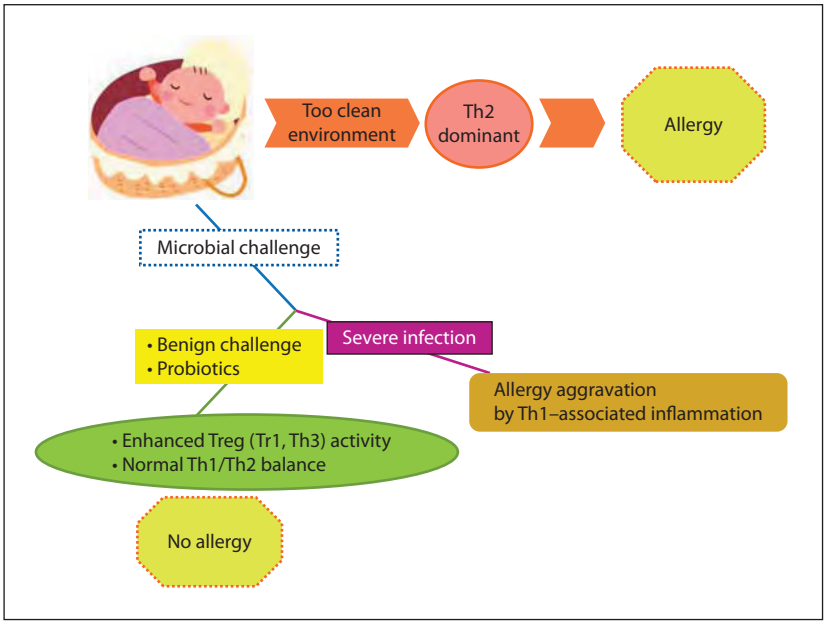

Fig. 1. Relationship between microbial challenge and occurrence of allergy.

those eating mainly sterilized foods. Recently, the administration of probiotic bacteria was reported to help maintain anti-inflammatory and tolerant immunity, which led to the lower prevalence of allergy in the subjects. In our double-blind, randomized placebo-controlled human trial, infants who were perinatally administered a combination of *B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031 showed significantly lower prevalence and cumulative incidence of AD than the placebo group [unpubl. data]. Hattori et al. [8] reported that the children with AD and low number of intestinal *Bifidobacterium* colonization showed amelioration of allergic symptoms when lyophilized *Bifidobacterium* was administered orally. In a double-blind, randomized, placebo-controlled trial, prenatal supplementation of *Lactobacillus reuteri* ATCC 55730 (1×10^8 colony forming units daily) in mothers from gestational week 36 until delivery and subsequent postnatal supplementation in babies from birth until 12 months of age resulted in less IgE-associated eczema at 2-year follow-up, although a preventive effect of probiotics on infant eczema was not confirmed [9]. The oral administration of combined *L. rhamnosus* and *L. reuteri* improved the extent of the eczema and decreased serum eosinophil cationic protein levels in children [10]. The effect was more pronounced in patients with a positive skin prick test response and elevated IgE levels. Supplementation of *B. lactis* Bb-12 or *L. rhamnosus* GG to the infants with atopic eczema during the weaning period reduced the extent and severity of atopic eczema, which was accompanied by the reduction in serum CD4 and urine eosinophilic protein X. Results from the above two clinical studies suggest that

probiotics may improve the symptoms of inflammatory responses in allergic diseases beyond the intestinal milieu [11]. An increased traffic of circulating CD34+ hemopoietic precursor cells has been suggested to be a key factor in systemic allergic inflammation [12]. In the 14 6- to 48-year-old allergic patients with clinical symptoms of asthma and/or conjunctivitis, rhinitis, urticaria, AD, food allergy and irritable bowel syndrome, the number of circulating CD34+ hemopoietic precursor cells was decreased when a mixture of *L. acidophilus*, *L. delbrueckii*, and *Streptococcus thermophilus* was administered for 30 days [12]. The possibility of transferring immune regulatory cytokine from mother to infant through mother's milk was suggested. When 62 pairs of mother and infant were supplemented with probiotics during pregnancy and the breastfeeding period, the level of TGF-β2 was higher in the breast milk from mothers in the probiotics group than from the control group mothers. The incidence of atopic eczema was significantly lower in infants born to the probiotics group mothers even at 2 years after delivery [13]. This result suggested that probiotics administered to mothers during pregnancy or breast feeding period increased the immune protective ability of the mother's milk and contributed to the protection of infants from suffering atopic eczema. Positive clinical effects of *L. rhamnosus* GG on the prevention of AD in infants gained attention of the scientific community and generated enthusiasm to use probiotics for the prevention and treatment of various diseases related to the allergy. When *L. rhamnosus* GG was administered to pregnant mothers and subsequently to infants after delivery, the incidence of AD was reduced by half in comparison with the placebo group [14]. However, a more recent study which employed almost identical study design showed that supplementation of *Lactobacillus* GG (5×10^9 CFU twice daily during pregnancy and early infancy) did not reduce the incidence or severity of AD in affected children. Instead, it was associated with an increased rate of recurrent episodes of wheezing bronchitis [15]. The effect of different probiotics on children with milk allergy was compared between those fed *L. rhamnosus* GG and those fed mixed probiotics with 4 strains. Levels of plasma IL-2, IL-4, IL-6, IL-1, TNF-α, TGF-β1, TGF-β2 and C-reactive protein were compared. Among them, levels of C-reactive protein and IL-6 were higher in the *L. rhamnosus* GG group and the level of IL-10 was higher in the probiotic mixture group. As C-reactive protein and IL-6 are inflammatory cytokine markers and IL-10 is an immune suppressive regulatory factor, the administration of *L. rhamnosus* GG was thought to increase inflammatory immunity. The potential inflammatory nature of *L. rhamnosus* was apparent in its association with endocarditis and liver abscess in very rare cases [16, 17]. Therefore, despite the considerable number of reports on the beneficial effect of *L. rhamnosus* GG on health, caution is warranted for consumption of *L. rhamnosus* GG by the patient with infectious or inflammatory diseases. More recently, it has been reported that there is an increased risk of mortality in patients with predicted severe acute pancreatitis who use prophylactic probiotics [18]. Another negative result of *Lactobacillus* use was reported. In a randomized controlled trial, supplementation of *L. acidophilus* (LAVRI-A1) for the first 6 months of life did not reduce the risk of AD

in high-risk infants but was associated with increased risk of subsequent cow's milk sensitization [19].

Recently, two meta-analyses of clinical trials on the prevention and treatment of pediatric allergic diseases by the administration of probiotics have been published. Osborn and Sinn's [20] meta-analysis of five studies reporting the outcomes of 1,477 infants showed a significant reduction in infant eczema but no significant reduction in atopic eczema confirmed by the skin prick test or specific IgE, though there was significant and substantial heterogeneity between studies. Lee et al. [21] conducted a meta-analysis of 10 double-blind randomized controlled clinical trials which pooled data from 6 prevention studies (n = 1,581) and 4 treatment trials (n = 299) by using fixed effects and random effects models of relative risk ratios and of weighted mean difference, respectively. The results supported the preventive effect of probiotics on pediatric AD but not the treatment effect.

As described above, most of the studies on the prevention of allergic diseases have been conducted in infants or children with AD symptoms. A decrease in allergic symptoms and lower levels of total IgE throughout the year were also shown in the elderly people (55–70 years old) consuming yogurt with live culture for 1 year but not in those consuming pasteurized yogurt [22]. However, further studies with well-designed, placebo-controlled clinical protocols are needed to determine whether any specific probiotic strain may be useful in the management of allergic symptoms in the senior people.

Current scientific evidence for the effect of probiotics on primary prevention of allergy is not conclusive but promising. The conflicting results may be due to the differences in study design, host and environmental factors, number and strain of the applied probiotics, which lead to the difficulties in direct comparison between the results from the different studies. Even though the suppression of the occurrence of allergy by probiotics has been documented in human studies, their mechanism of action on the regulation of immune system is not well known. The primary action of probiotics might be through direct contact with GI lymphoid tissue. However, the indirect action of probiotics might also play a partial role in the suppression of allergy. For instance, probiotics might be able to reduce the number of harmful GI bacteria which can cause inflammatory reaction or degrade mucous cell layer and thereby aggravate the allergic reaction or increase permeability of the allergen through the GI epithelial cell layer. Administration of live *Bifidobacterium* cells was more effective in the suppression of allergy than heat-treated cells or sonicated fragmented cells in the ovalbumin (OVA) allergy animal model [23]. The live cells might have suppressed other harmful intestinal bacteria more effectively or the number of administered bacteria might have increased after colonization in the intestine resulting in the higher dose of active material. The permeability of the intestine was thought to be closely related to the occurrence of allergy. Administration of *L. rhamnosus* and *L. reuteri* in children with AD for 6–41 weeks improved GI symptoms and AD and decreased lactulose/mannitol permeability from the lumen to the blood, suggesting that

administration of probiotics may reduce gut permeability in atopic children [24]. Use of probiotic bacteria was suggested for the management of various diseases associated with increased gut permeability due to impaired gut barrier or intestinal inflammation associated with acute rotavirus diarrhea and various colonic disorders as well as food allergy [25]. If direct interaction of probiotics is the primary mechanism in the prevention of allergic diseases, early infant period would be the most important stage for intervention since the administered probiotics can get access to the yet immature gut immune cells and play a role in their maturation process. Accordingly, most of the allergy preventive effects of the probiotics were shown in infants. In mice, the amount of luminal secretory IgA drastically decreased around the weaning period and presumably transport of the bacterial antigens to the vulnerable immune inductive site was facilitated, thus providing an opportunity for the probiotics to modulate the host immune system via gut immune system and to induce tolerance against allergies [26]. Consequently, the primary administration of *L. johnsonii* La1 during specific window of weaning period was effective in the prevention of AD manifested at the systemic level.

Effect of Probiotics in Animal Models or in Cell Culture Assays

Gut-associated lymphoid tissue contains Peyer's patches (PPs) and isolated lymphoid follicles, inductor site of immune responses, and the larmina propria, effector site of immune responses. These immune tissues contain B cells, T cells, dendritic cells (DCs), and macrophages. DCs or/and T-regulatory cells are suggested to play a crucial role in establishing a tolerance in both mucosal and systemic immunity [26–28]. Gut epithelial cells, lymphoid cells, and DCs constantly recognize and interact with bacterial cells or their components such as peptidoglycan, lipoprotein, and lipopolysaccharide using pattern receptor system, including toll-like receptors (TLR), and mediate innate or adaptive immune responses [29]. At the intestinal mucosal layer, probiotic bacteria and their cell components such as peptidoglycan, lipoteichoic acid, intra- and extracellular polysaccharide products, cell-free extracts, and cell walls have been reported to cross-talk with the intestinal epithelial cells, M cells in PPs, and underlying DCs and macrophage cells. The multiple consequences of the cross-talk between the probiotic bacteria and the intestinal mucosa lead to the reinforcement of the intestinal barrier as well as direct modulation of mucosal immune cell functions including cytokine and chemokine release (fig. 2) [30].

Oral intake of the *L. pentosus* strain induced IFN-γ-producing cells through activation of IL-12 production by CD11c+DCs in a TLR 2- and/or a TLR4-dependent manner. This was confirmed by the observation that the production of IL-12p70 by DCs and IL-12p70 and IFN-γ production by spleen cells significantly decreased in those cells isolated from $TLR2^{-/-}$ or $TLR4^{-/-}$ mice compared with those from wild-type mice [31]. In comparison, the suppressive effect of CpG oligodexoynucleotide

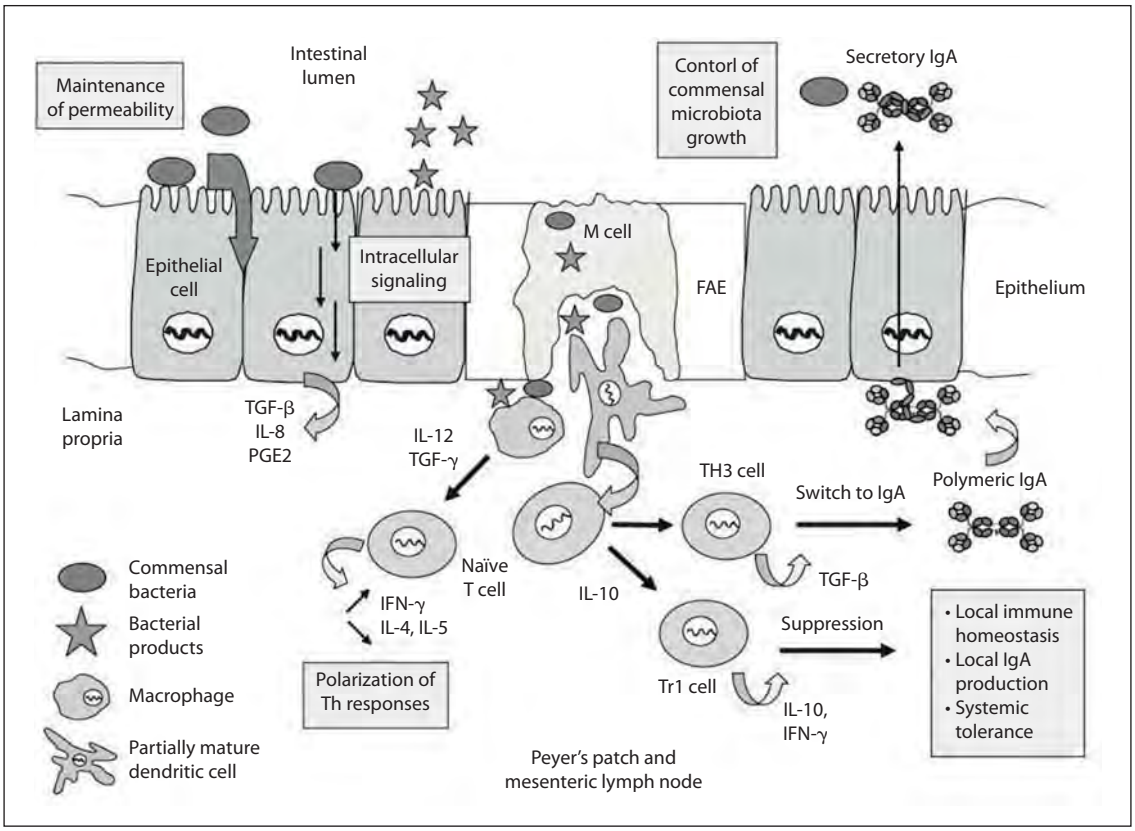

Fig. 2. Schematic representation of multiple consequences of cross-talk between probiotic bacteria and intestinal mucosa [30].

from *L. rhamnosus* GG on antigen-specific IgE production in OVA-sensitized mouse model was suggested to be dependent on TLR9 in CD11c+CD8+ DCs [32]. The coapplication of *L. plantarum* and Der p 1, the major house dust mite allergen of *Dermatophagoides pteronyssinus*, suppressed specific IgE response and favored the production of INF-γ upon allergen restimulation. This strain was shown to stimulate high IL-12 and moderate IL-10 production in mouse DCs derived from the bone marrow notably through the TLR2-, MyD88-dependent and TLR4-independent pathway [33]. These different results suggest that the interaction of probiotics and DCs are differentially affected by discrete components of the different strains and the types of TLR in DCs. Although the interaction of DCs and probiotics may play a primary and pivotal role, several strains of lactobacilli and bifidobacteria are known to influence immune function through a number of different pathways including effects on enterocytes, antigen-presenting cells including circulating monocytes, DCs, regulatory T cells, and effector T and B cells [34]. DCs that have sampled dietary, self or commensal bacterial antigens may go on to induce tolerogenic T cell responses upon

migration to the mesenteric lymph nodes [35]. Activation of T cells is regarded as a property of mature DCs that have travelled to draining lymphoid tissue and express high levels of surface MHC class II and costimulatory molecules. Veckman et al. [36] showed that certain probiotics directly enhance the activity of human DC populations to express moderate levels of costimulatory molecules and cytokines thereby facilitating Th1 cell differentiation. Additionally, probiotics induced an increase in regulatory DCs and T cells which then led to immunoregulatory mechanisms mediated in part by release of IL-10 and TGF-β [37]. IL-10 is known to be involved in the activation of Tr1 type regulatory T cells. TGF-β is known to be an important factor to enhance the differentiation of regulatory Th3 cells. Although many in vitro studies in which experimental probiotics were arranged to interact with a specific type of immune cell have provided useful information on their immunomodulatory actions; oftentimes, these in vitro results were contradictory to what have been observed in in vivo studies. Therefore, caution is warranted so that in vitro culture results do not lead to the error-prone interpretation of the role of probiotics in in vivo immune regulation. To compensate the weakness of a simple in vitro experiment, we adopted various transwell coculture systems in which epithelial cells were grown in the insert layer and other cell types such as DCs, spleen cells, or PP cells were grown on the bottom layer [unpubl. data]. In this cocultured experiment with epithelial and DC lines, various probiotic strains either slightly decreased or did not affect the expression of I-A^d, CD86, CD40, and the levels of IL-6 and TNF-α produced. Interestingly, *B. lactis* increased IL-10 secretion, and *L. casei* and *L. acidophilus* increased TGF-β secretion. Further development of a more elaborate in vitro model will help in understanding the role of the probiotics in the regulation of the immune system in relation to their differences in strain, dose, route and timing of exposure.

Various in vivo animal experimental models have been employed to characterize and assess how probiotics modulate an allergy-related immune system. Animal models may provide more useful information on the mechanisms of the antiallergic effects of probiotic bacteria than in vitro studies. Depending on the protocols of the animal models, experimental results suggest that the administration of probiotics either potentiate Th1 relative to Th2 or enhance T regulatory cell activity. Orally administered *L. casei* reduced antigen-specific contact skin sensitivity by controlling the size of the CD8+ effector pool, which was mediated by regulatory CD4+ T cells [38]. Even though several other studies showed that the suppression of antigen-specific IgE by the administration of lactic acid bacteria was associated with the enhanced secretion of Th1-associated cytokines and low levels of Th2-associated cytokines, those studies in which the experimental allergens or probiotic strains were intraperitoneally injected were not included for review in the present study. These experimental models based on intraperitoneal injection do not reflect the real route of entry of antigen or experimental probiotic strain into the human body, and thus make the interpretation of the real mechanism involved difficult. Oral administration of both probiotics and a specific antigen may be more desirable. Several studies

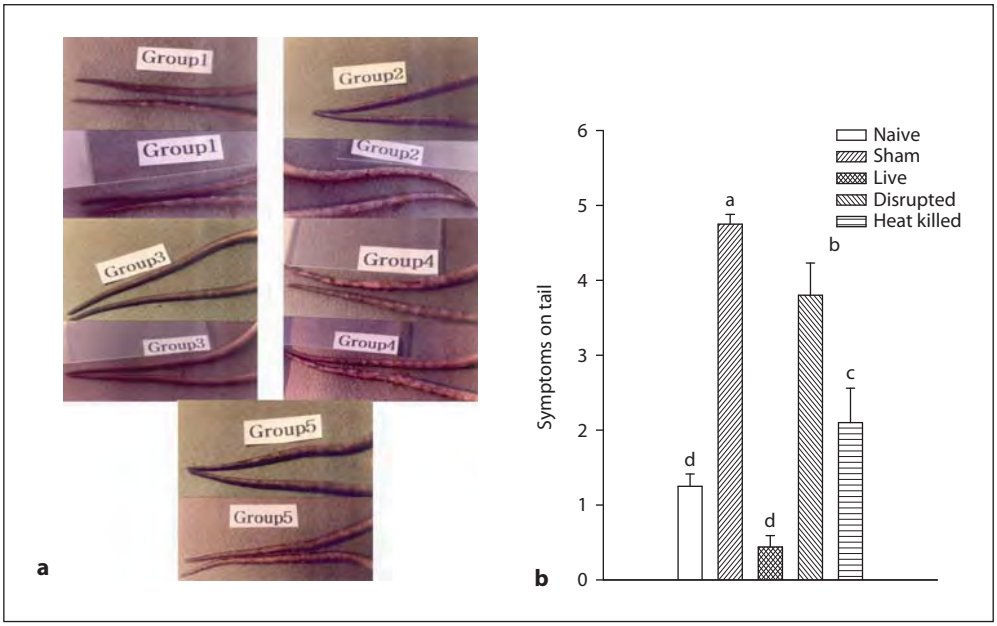

Fig. 3. Severity of allergic symptoms on the tails of OVA-sensitized mice treated with various components of *B. bifidum* BGN4. **a** Photographs of the tails of the experimental mice. Group 1, naive; group 2, sham; group 3, treated with live BGN4; group 4, treated with disrupted BGN4; group 5, treated with heat-killed BGN4. **b** Symptom scores of the tails of the experimental mice. Different letters indicate significant differences in Duncan's multiple range tests ($p < 0.05$) [23].

assessed the effect of orally administered probiotics in animals which were sensitized to develop allergic symptoms to oral OVA challenge. In this model, oral administration of probiotics suppressed production of the OVA-specific IgE, IgG1 in serum, OVA-specific fecal IgA, and the level of splenic IL-4 production and enhanced the production of splenic INF-γ and IL-10. In addition, the groups treated with probiotics showed ameliorated tail scabs and lower levels of degranulated mast cells in ears and small intestines, and infiltrated eosinophil granules in small intestines [23, 39]. Viable *Bifidobacterium* was more effective than disrupted or heat-killed cells in suppressing the symptoms of allergy (fig. 3). Antiallergic effects of the probiotics seemed to be manifested at the local and systemic levels and also at the initial and later phases during allergic progression. The observed suppression of IL-4 might be indicative of the potentiation of Th1 cells, and enhanced IL-10 secretion might have partially contributed to the induction of oral tolerance by activating regulatory T cells. Torii et al. [40] showed that oral administration of *L. acidophilus* L-92 to OVA-induced allergy mice suppressed OVA-specific IgE production and cytokines such as IFN-γ, IL-4 and IL-10. Additionally, antibodies such as total IgE and OVA-specific IgE were produced at a significantly lower level in the splenocytes of L-92-treated mice than those of control mice. In contrast, TGF-β and IgA levels produced by PPs from L-92-treated

mice were significantly higher than those produced by control mice. In addition, a specific IgE-suppressive effect of *B. bifidum* G9-1 was suggested to be mediated by Treg cells independent of IFN-γ production [41]. Although the results of the animal experiment were not exactly similar, most of the studies advocated the use of probiotics for the alleviation of allergy. Taken together, oral administration of probiotics has been demonstrated to induce protective immune responses against allergic symptoms at local and systemic levels in animal models and human studies and might provide a rationale to utilize probiotics in the prevention of allergic diseases during the infantile period in humans.

Acknowledgment

This work was supported by a grant (A080664) from the Ministry for Health, Welfare and Family Affairs, Republic of Korea.

References

1 Williams H, Robertson C, Stewart A, Ait-Khaled N, Anabwani G, Anderson R, Asher I, Beasley R, Bjorksten B, Burr M, Clayton T, Crane J, Ellwood P., Keil U, Lai C, Mallol J, Martinez F, Mitchell E, Montefort S, Pearce N, Shah J, Sibbald B, Strachan D, von Mutius E, Weiland SK: Worldwide variations in the prevalence of symptoms of atopic eczema in the international study of asthma and allergies in childhood. J Allergy Clin Immunol 1999;103:125–138.

2 Strachan DP. Hay fever, hygiene, and household size: Br Med J 1989;299,1259–1260.

3 Flohr C, Pascoe D, Williams HC: Atopic dermatitis and the hygiene hypothesis: too clean to be true. British J Dermatol 2005;152:202–16.

4 Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y: The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol 1997;159:1739–1745.

5 Rautava S, Ruuskanen O, Ouwehand A, Salminen S, Isolauri E: The hygiene hypothesis of atopic disease-An extended version. J Pediatr Gastroenterol Nutr 2004;38:378–388.

6 Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M: Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol 2001;108:516–520.

7 Alm JS, Swartz J, Lilja G, Scheynius A, Pershagen G: Atopy in children of families with an anthroposophic lifestyle. Lancet 1999;353:1485–1488.

8 Hattori K, Yamamoto A, Sasai M, Taniuchi S, Kojima T, Kobayashi Y, Iwamoto H, Namba K, Yaeshima T: Effects of administration of bifidobacteria on fecal microflora and clinical symptoms in infants with atopic dermatitis. Arerugi 2003;52:20–30.

9 Abrahamsson TR, Jakobsson T, Böttcher MF, Fredrikson M, Jenmalm MC, Björkstén B, Oldaeus G: Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol. 2007;119:1174–1180.

10 Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH, Paerregarrd A: Effect of probiotic Lactobacillus strains in children with atopic dermatitis. J Allergy Clin Immunol 2003;111:389–395.

11 Isolauri E, Arvola T, Stas Y, Salminen S: Probiotics in the management of atopic eczema. Clin Exp Allergy 2000;30:1605–1610.

12 Mastrandrea F, Conrdduzza G, Serio G, Minardi A, Manelli M, Ardito S, muratore L: Probiotics reduce the CD34+ hemopoietic precursor cell increased traffic in allergic subjects. Eur Ann Allergy Clin Immunol 2004;36:118–122.

13 Rautava S, Kalliomki M, Isolauri E: Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. J Allergy Clin Immunol 2002;109:119–121.

14 Kallomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E: Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. Lancet 2001;357:1076–1079.
15 Kopp MV, Hennemuth I, Heinzmann A, Urbanek R: Randomized, double-blind, placebo-controlled trial of probiotics for primary prevention: no clinical effects of *Lactobacillus* GG supplementation. Pediatrics 2008;121:e858–856
16 Presterl E, Kneifel W, Mayer HK, Zehetgruber M, Makristathis A, Graninger W: Endocarditis by *Lactobacillus rhamnosus* due to yogurt ingestion? Scand J Infect Dis 2001;33:710–714.
17 Rautio M, Jousimies-Somer H, Kauma H, Pietarinen I, Saxelin M, Tynkkynen S, Koskela M: Liver abscess due to a *Lactobacillus rhamnosus* strain indistinguishable from *L. rhamnosus* strain GG.. Clin Infect Dis 1999;28:1159–1160.
18 Besselink MG, van Santvoort HC, Buskens E, Boermeester MA, van Goor H, Timmerman HM, Nieuwenhuijs VB, Bollen TL, van Ramshorst B, Witteman BJ, Rosman C, Ploeg RJ, Brink MA, Schaapherder AF, Dejong CH, Wahab PJ, van Laarhoven CJ, van der Harst E, van Eijck CH, Cuesta MA, Akkermans LM, Gooszen HG: Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. Lancet 2008;371:651–659.
19 Taylor AL, Dunstan JA, Prescott SL: Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: A randomized controlled trial. J Allergy Clin Immunol. 2007;119:184–191.
20 Osborn DA, Sinn JK: Probiotics in infants for prevention of allergic disease and food hypersensitivity. Cochrane Database Syst Rev 2007;17,CD006475.
21 Lee J, Seto D, Bielory L: Meta-analysis of clinical trials of probiotics for prevention and treatment of pediatric atopic dermatitis. J Allergy Clin Immunol 2008;121, 116–121
22 Van de Water J, Keen CL, Gershwin ME: The influence of chronic yogurt consumption on immunity. J Nutr 1999;129 Suppl 7:1492–1495.
23 Kim H, Ji GE: Effect of viability and integrity of *Bifidobacterium* on suppression of allergy in mice. J Microbiol Biotechnol 2006;16:1010–1016.
24 Rosenfeldt V, Benfeldt E, Valerius NH, Paerregaard A, Michaelsen KF: Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. J Pediatr 2004;145:612–616.
25 Salminen S, Isolauri E, Salminen E: Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Van Leeuwenhoek 1996;70, 347–358.
26 Inoue R, Nishio A, Fukushima Y, Ushida K: Oral treatment with probiotic *Lactobacillus johnsonii* NCC533 (La1) for a specific part of the weaning period prevents the development of atopic dermatitis induced after maturation in model mice, NC/Nga. Br J Dermatol 2007;156:499–509.
27 Williamson E, Westrich GM, Viney JL: Modulating dendritic cells to optimize mucosal immunization protocols. J Immunol 1999;163:3668–3675.
28 Sakaguchi S: Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000;101:455–458.
29 Blander JM, Medzhitov R: Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature 2006;440:808–812.
30 Corthésy B, Gaskins HR, Mercenier A: Cross-talk between probiotic bacteria and the host immune system. J Nutr 2007;137:781S-790S.
31 Koizumi S, Wakita D, Sato T, Mitamura R, Izumo T, Shibata H, Kiso Y, Chamoto K, Togashi Y, Kitamura H, Nishimura T: Essential role of Toll-like receptors for dendritic cell and NK1.1(+) cell-dependent activation of type 1 immunity by *Lactobacillus pentosus* strain S-PT84. Immunol Lett 2008;120:14–19.
32 Iliev ID, Tohno M, Kurosaki D, Shimosato T, He F, Hosoda M, Saito T, Kitazawa H: Immunostimulatory oligodeoxynucleotide containing TTTCGTTT motif from *Lactobacillus rhamnosus* GG DNA potentially suppresses OVA-specific IgE production in mice. Scand J Immunol 2008;67:370–376.
33 Hisbergues M, Magi M, Rigaux P, Steuve J, Garcia L, Goudercourt D, Pot B, Pestel J, Jacquet A: In vivo and in vitro immunomodulation of Der p 1 allergen-specific response by *Lactobacillus plantarum* bacteria Clin Exp Allergy 2007;37:1286–95.
34 Prescott SL, Bjorksten B: Probiotics for the prevention or treatment of allergic diseases. J Allergy Clin Immunol 2007;120:255–262.
35 Scheinecker C, McHugh R, Shevach EM, Germain RN: Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J Exp Med 2002;196:1079–1090.
36 Veckman V, Miettinen M, Pirhonen J, Siren J, Matikainen S, Julkunen I: *Streptococcus pyogenes* and *Lactobacillus rhamnosus* differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells. J Leukoc Biol 2004;75:764–771.
37 Rook GAW, Brunet LR: Microbes, immunoregulation, and the gut. Gut 2005;54:317–320.
38 Chapat L, Chemin K, Dubois B, Bourdet-Sicard R, Kaiserlian D: *Lactobacillus casei* reduces CD8+ T cell-mediated skin inflammation. Eur J Immunol 2004;34:2520–2528.

39 Kim H, Kwack K, Kim DY, Ji GE: Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model. FEMS Immunol Med Mic 2005;45:259–267.
40 Torii A, Torii S, Fujiwara S, Tanaka H, Inagaki N, Nagai H: *Lactobacillus acidophilus* strain L-92 regulates the production of Th1 cytokine as well as Th2 cytokines. Allergol Int 2007;56:293–301.
41 Ohno H, Tsunemine S, Isa Y, Shimakawa M, Yamamura H: Oral administration of *Bifidobacterium bifidum* G9–1 suppresses total and antigen specific immunoglobulin E production in mice. Biol Pharm Bull 2005;28:1462–1466.

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Review article
http://dx.doi.org/10.3345/kjp.2012.55.6.193
Korean J Pediatr 2012;55(6):193-201
eISSN 1738-1061 • pISSN 2092-7258

Effects of probiotics on the prevention of atopic dermatitis

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Atopic dermatitis (AD) is an immune disorder that is becoming increasingly prevalent throughout the world. The exact etiology of AD remains unknown, and a cure for AD is not currently available. The hypothesis that appropriate early microbial stimulation contributes to the establishment of a balanced immune system in terms of T helper type Th1, Th2, and regulatory T cell (Treg) responses has led to the use of probiotics for the prevention and treatment of AD in light of various human clinical studies and animal experiments. Meta-analysis data suggests that probiotics can alleviate the symptoms of AD in infants. The effects of balancing Th1/Th2 immunity and enhancing Treg activity via the interaction of probiotics with dendritic cells have been described *in vitro* and in animal models, although such an effect has not been demonstrated in human studies. In this review, we present some highlights of the immunomodulatory effects of probiotics in humans and animal studies with regard to their effects on the prevention of AD.

Key words: Probiotics, Dermatitis, Atopic, Prevention, Immunomodulation, Immune system

Introduction

Atopic dermatitis (AD) is a common, chronic, and refractory skin disease manifesting as eczema and pruritus with repeated exacerbations and regressions[1]. The prevalence of allergic diseases such as AD, asthma, and allergic rhinitis (AR) has increased throughout the world during the last 30 years, with the cumulative prevalence of AD in children reaching 8 to 20%[2], although the eczema symptom prevalence for children 13 to 14 years of age has decreased in some previously high-prevalence areas in the developed world, such as the United Kingdom and New Zealand[3]. The prevalence of allergy tends to be lower in a family with a higher number of siblings; the incidence of allergic diseases increases when one moves from a low-prevalence area to a high-prevalence area and is higher in urban areas than in rural areas. These observations emphasize the importance of environmental factors in the development of allergic diseases, as well the role of genetic factors and the maturity of epithelial cell barrier functions[4]. A systematic review found no strong evidence of a protective effect of exclusive breastfeeding for at least 3 months against eczema, even in those with a positive family history of atopy[5]. Environmental factors that may contribute to the increased development of AD include decreased microbial exposure due to urbanization, the use of vaccines and antibiotics, and improved infant sanitation. Exposure of infants to farm animals, pets, and daycare environments is known to be helpful for the introduction of benign challenges, including various non-pathogenic microorganisms,

which leads to the establishment of protective immunity against allergic disorders[4]. The intestinal immune system comprises the largest portion of the overall immune system and remains exposed to intestinal bacteria, thus accounting for the largest source of microbial exposure in humans. The immune system of neonatal infants is not fully developed and is prone to an immature T helper type Th2-dominant state. Infants undergo environment-driven maturation to establish a balance between Th1, Th2, Th17, and regulatory T cell (Treg) responses; appropriate microbial stimulation in early life contributes to the establishment of a balanced immune system[6]. In AD, the onset of acute skin lesions is initiated by Th2-dominant cells. If this initial Th2 cellular response is not suppressed, a subsequent Th1 response is induced, consisting of inflammatory reactions resulting in chronic inflammation due to the secretion of pro-inflammatory cytokines by dying keratinocytes[7].

The effect of probiotics in human studies

Probiotics administered in sufficient amounts can have a beneficial effect on the health of the host[8]. Probiotics can relieve travelers' diarrhea, antibiotic-associated diarrhea, atopic eczema, and irritable bowel syndrome (IBS)[9]. Infants with AD or other allergic diseases show less frequent intestinal colonization with probiotics such as *Lactobacillus* or *Bifidobacterium* and more frequent colonization with *Clostridium* relative to non-allergic infants[10]. Recently, the administration of probiotic bacteria was reported to help maintain anti-inflammatory and tolerant immunity, which resulted in a lower prevalence of allergy in human subjects (Table 1). Potential mechanisms explaining the health-promoting actions of probiotic bacteria may include modulation of the intestinal immune system and displacement of potential pathogens via competitive exclusion or the production of antimicrobial agents. The efficacy of probiotics in the prevention of AD was primarily observed in infants who were administered probiotics during the perinatal period. Hattori et al.[31] reported that children with AD and with a low degree of intestinal *Bifidobacterium* colonization experienced an amelioration of their allergic symptoms when lyophilized *Bifidobacterium* was administered orally. Systematic analysis in some clinical studies suggested that the intake of probiotics by mothers during pregnancy reduces the incidence of subsequent eczema[5]. Young children with immunoglobulin E (IgE)-mediated atopic eczema, in particular, showed more significant improvement with the administration of probiotics[32]. Other studies have also shown that the adult immune system is more difficult to modulate by the administration of probiotics. An analysis of systematic reviews suggests that there is little evidence to support a clinically useful benefit of using probiotics

Table 1. The Effects of Probiotics on Allergic Diseases in Human Clinical Trials (since 2009)

Year	Group	Strains (formula)	Definition of subjects completed the trial	Intervention method	Dose	Time of treatment	Outcome
2012	Gore, et al.[11]	*L. paracasei* CNCM I- 2116 or *B. lactis* CNCM I-3446	137 Infants with eczema at age 3-6 months	Placebo-control trials	10^{10} CFUs of either lyophilized powdered CNCM I-2116 or CNCM I-3446 daily	12 Weeks	No effect on treatment and secondary prevention effects
2011	Roessler, et al.[12]	A probiotic mixture (*L. paracasei* Lpc-37, *L. acidophilus* 74-2, *B. animalis* subsp. *lactis* DGCC 420)	15 Healthy adults,15 patients with AD	A randomized, placebo-controlled study	2×100 mL/day of either a probiotic or a placebo drink	8 Weeks	Reduction of genotoxic activity of faecal water in patients with AD
2011	Larsen, et al.[13]	*L. acidophilus* NCFM, *B. lactis* Bi-07	50 Young children with AD	RDC	10^{10} CFU daily	8 Weeks	No affect on the composition and diversity of the main bacterial populations in feces
2011	Morisset, et al.[14]	A non-hydrolyzed, fermented milk formula containing heat-killed *B. breve* C50, *S. thermophilus* 065 (HKBBST milk)	After birth until 2-year-old, 124 children at high risk of atopy	RDC	*B. breve* C50 (4.2×10^{9} bacteria per 100 g of powder formula), *S. thermophilus* 065 (3.84×10^{7} bacteria per 100 g of powder formula) daily	12 Months	Reduction of digestive and respiratory events
2011	Torii, et al.[15]	*L. acidophilus* L-92 (L-92)	Japanese children with AD	A double-blinded, placebo-controlled study	–	Long-term administration	Shown a complementary effect on the standard medical therapy in patients with AD through the alteration in the Th1/Th2
2011	Boyle, et al.[16]	*L. rhamnosus* GG (LGG)	250 Pregnant women carrying infants at high risk of allergic disease	RCT	LGG (1.8×10^{10} CFUs) daily	From 36 weeks gestation until delivery	Not sufficient for preventing eczema

Table 1. The Effects of Probiotics on Allergic Diseases in Human Clinical Trials (since 2009) (Continued)

Year	Group	Strains (formula)	Definition of subjects completed the trial	Intervention method	Dose	Time of treatment	Outcome
2011	Kukkonen, et al.[17]	*L. rhamnosus* GG (ATCC53103), *L. rhamnosus* LC705 (DSM7061), *B. breve* Bb99 (DSM13692), *P. freudenreichii* ssp.*shermanii* JS (DSM7076)	1,018 Mothers with high risk and their 688 infants at age 2	RDC	*L.rhamnosus* GG (5×10^9 CFUs), *L.rhamnosus* LC705 (5×10^9 CFUs), *B. breve* Bb99 (2×10^8CFU), *P. freudenreichii* ssp. *shermanii* JS (2×10^9 CFUs), twice daily	From 36 weeks gestation after delivery 6 months	No immunomodulatory effect
2011	Nermes, et al.[18]	*L. rhamnosus* GG (LGG)	39 Infants with AD	A double-blind design	LGG (3.4×10^9 CFUs) daily	3 Months	Improvement of gut barrier function
2011	van der Aa, et al.[19]	A formula with *B. breve* M-16V, a galacto//fructooligosaccharide mixture (immunofortis)	75 Infants with AD, age<7 months	RDC, multicentre trial	–	12 Weeks	Preventive effect on the asthma-like symptoms in infants with AD
2011	Moroi, et al.[20]	Supplementarydiet containing heat-killed lactic acid bacterium *L. paracasei* K71	34 Adult patients with AD	RDC	–	>12 Weeks	Beneficial effect on adult type AD
2010	Hoang, et al.[21]	*L. rhamnosus* cell lysate	14 Patients (age 8-64 months) with a history resistant eczema for a period of at least 6 months	Open label non-randomized clinical observation	300-500 mg standardized *L. rhamnosus* cell lysate daily	–	Effective on the treatment and prevention of childhood eczema
2010	Dotterud, et al.[22]	Probiotic milk containing *L. rhamnosus* GG, *L. acidophilus* La-5, *B. animalis* subsp. *lactis* Bb-12 (Biola)	Children from a non selected maternal, women from 36 weeks of gestation to 3 months postnatally during breastfeeding (pregnant women=415) (children =278)	Randomized, double-blind trial	250 mL probiotic low fat fermented milk (5×10^9 CFUs each bacterium) daily	From 36 weeks of gestation until 3 months postnatally	No effect on atopic sensitization
2010	Chen, et al.[23]	*L. gasseri* A5	10^5 Asthmatic children with AR (6-12 years)	RDC	One capsule (2×10^9 CFUs)	8 Weeks	Clinical benefits through the clinical symptom and immunological parameters including cytokine production
2010	Gerasimov, et al.[24]	*L. acidophilus* DDS-1, *B. lactis* UABLA-12 with fructo ligosaccharide	90 Children aged 1-3 years with moderate-to-severe AD	RDC	5 Billion CFUs twice daily	8 Weeks	Clinical improvement with corresponding lymphocyte subset changes in peripheral blood
2010	Rose, et al.[25]	*L. rhamnosus* GG	131 Children (6-24 months old) at high risk of allergic sensitization, asthma	Double-blind dietary supplement	10^{10} CFUs twice daily	>6 Months	No clinical effect
2010	Kim, et al.[26]	A probiotic mixture (*B. bifidum* BGN4, *B. lactis* AD011, *L. acidophilus* AD031)	112 Pregnant women with a family history of allergic diseases and their infants	RDC	BGN4 (1.6×10^9 CFUs), AD011 (1.6×10^9 CFUs), AD031 (1.6×10^9 CFUs) once daily	8 Weeks before the expected delivery to 3 months after delivery following breast-feeding until 3 months of age and formula-feeding of a probiotic mixture until 6 months of age	Effective on the primary prevention of eczema
2010	Woo, et al.[27]	*L. sakei* KCTC10755BP	75 Children aged 2-10 years with AEDS	RDC	Daily	12 Weeks	A substantial clinical improvement

Table 1. The Effects of Probiotics on Allergic Diseases in Human Clinical Trials (since 2009) (Continued)

Year	Group	Strains (formula)	Definition of subjects completed the trial	Intervention method	Dose	Time of treatment	Outcome
2009	Niers, et al.[28]	A probiotic mixture (*B. bifidum, B.lactis, Lactococcus lactis*; Ecologic Panda)	Pregnant women with a family history of allergic diseases and their infants	RDC	3×10^9 CFUs (1×10^9 CFUs of each strain) once daily	6 Weeks before the expected delivery to 12 months after delivery	Preventive effect on the incidence of eczema in high-risk children and maintenance during the first 2 years of life
2009	Soh, et al.[29]	*B. longum* (BL999), *L. rhamnosus* (LPR)	Asian infants at risk of allergic disease	RDC	BL999 (1×10^7 CFUs/g), LPR (2×10^7 CFUs/g) daily	For the first 6 months	No effect on prevention of eczema or allergen sensitization
2009	Kuitunen, et al.[30]	A probiotic mixture (2 lactobacilli, bifidobacteria, propionibacteria)	1,223 Mothers with infants at high risk for allergy	A double-blinded, placebo-controlled study	A capsule of LGG (5×10^9 CFUs), *L. rhamnosus* (5×10^9 CFUs), *B. breve* (2×10^8 CFUs), *P. freudenreichii* ssp. *shermanii* JS (2×10^9 CFUs) twice daily	During the last month of pregnancy and their infants to receive a probiotic mixture from birth until age 6 months	No allergy-preventive effect except in cesarean-delivered children

L., Lactobacillus; *B., Bifidobacterium*; S., *Streptococcus*; P., *Propionibacterium*; CFU, colony-forming unit; AD, atopic dermatitis; AR, allergic rhinitis; AEDS, atopic eczema-dermatitis syndrome; RDC, a randomized, double-blind, placebo-controlled study; RCT, a randomized, placebo-controlled trials.

in patients with established eczema, although immunomodulation may also occur in adulthood[5]. No beneficial effect was found from *B. lactis* or *L. paracasei* supplementation in the treatment of eczema when given as an adjunct to basic topical treatment, and no effect on the progression of allergic disease from age 1 to 3 years was noted[11]. The preventive effect of probiotics is strain specific; when *L. rhamnosus* HN001 and *B. animalis* subsp. *lactis* HN019 were compared, only *L. rhamnosus* HN001 reduced (by 2 years) the cumulative prevalence of eczema in infants at risk of allergic disease[33]. In various studies, the tested probiotic mixtures contained different strains, and individual strains were not tested. Therefore, it is still premature to conclude that individual component strain can have a synergistic effect when combined into a mixture, as comparisons of the effect of a probiotic mixture with that of one or more of its component strains have not been performed, particularly for severe diseases. Early life administration of a cow's milk formula supplemented with *B. longum* BL999 and *L. rhamnosus* LPR showed no effect on the prevention of eczema or allergen sensitization in the first year of life in infants at risk for allergic disease[29].

Occasionally, an identical strain shows contradictory results. In the case of *L. rhamnosus* GG, early reports suggested that *L. rhamnosus* GG (1×10^{10} colony-forming units [CFUs] of *L. rhamnosus* GG daily) administered to pregnant mothers and subsequently to infants after delivery reduced the incidence of AD by half relative to those treated with placebo[34]. However, a more recent study that employed a nearly identical study design showed that supplementation of *L. rhamnosus* GG (5×10^9 CFUs twice daily during pregnancy and early infancy) did not reduce the incidence and the severity of AD in affected children. Rather, probiotic supplementation was associated with an increased rate of recurrent episodes of wheezing bronchitis[35]. Moreover, oral administration of *L. rhamnosus* GG in a prospective, double-blind, randomized, placebo-controlled study had no clinical effect on AD or asthma-related events in young children (6 to 24 months old) with recurrent wheezing and a family history of atopy[25].

Although a meta-analysis revealed the positive potential of probiotics, the mechanism of action or biomarkers related to their anti-AD effect were not clarified. The reduction of AD prevalence in infants with a family history of allergic diseases by the administration of a probiotic mixture (*B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031) was associated with significant increases in the capacity of transforming growth factor beta (TGF-β) production by peripheral blood mononuclear cells[36]. When 62 mother and infant pairs were supplemented with probiotics during pregnancy and their breastfeeding period, the level of TGF-β_2 was higher in the breast milk from mothers in the probiotics group than in that from mothers in the control group[37]. Compared with a placebo, the administration of *L. sakei* KCTC 10755BP to children aged 2 to 10 years with atopic eczema-dermatitis syndrome and a minimum SCORing of Atopic Dermatitis (SCORAD) score of 25 resulted in a decreased SCORAD total score associated with lower pretreatment-adjusted serum levels of chemokine (c-c motif) ligand CCL17 and CCL27, which are chemokines involved in the process of establishing inflammatory infiltration of cells[27]. Oral administration of combined *L. rhamnosus* and *L. reuteri* improved the extent of the eczema and decreased serum eosinophil cationic protein levels in children[38]; this effect was more pronounced in patients with a positive skin prick test response and elevated IgE levels. Supplementation of *B. lactis* Bb-12 or *L. rhamnosus* GG to infants with atopic eczema during the weaning

period reduced the extent and the severity of atopic eczema, which was accompanied by the reduction of serum cluster of differentiation (CD)4 and urine eosinophilic protein X[39]. Taken together, the results from the 3 clinical studies described above suggest that probiotics improve the symptoms of inflammatory responses in allergic diseases beyond the intestinal milieu[39]. An increase in the traffic of circulating CD34+ hematopoietic precursor cells (HPCs) was suggested to be a factor in systemic allergic inflammation[40]. In 14 allergic patients who were 6 to 48 years old with clinical symptoms of asthma and/or conjunctivitis, rhinitis, urticaria, AD, food allergy, and IBS, the number of circulating CD34+ HPC was decreased when a mixture of *L. acidophilus*, *L. delbrueckii*, and *Streptococcus thermophilus* was administered for 30 days[40]. A study to assess whether the administration of probiotics affects the microbiota and its genotoxic activity in healthy subjects and patients with AD revealed that the administration of a probiotic mix containing *L. paracasei* Lpc-37, *L. acidophilus* 74-2, and *B. animalis* subsp. *lactis* DGCC 420 decreased the genotoxic potential of fecal water in AD patients. The fecal *C. perfringens* cluster I-II levels remained unaffected, suggesting either a change in their activity or that other bacterial species are responsible for the reduced genotoxic activity of fecal water[12]. In an allergic condition, the function of Tregs and their production of cytokines such as interleukin (IL)-10 and TGF-β are dysregulated compared to the normal condition, resulting in prolonged inflammatory responses against environmental allergens[41]. The intestinal epithelial cells secrete thymic stromal lymphopoietin, TGF-β, and retinoic acid, which induce the development of resident CD11b regulatory dendritic cells (DCs), which in turn induce the development of naive T cells into forkhead box family transcription factor Foxp3$^+$ Tregs[42]. One plausible reason as to why the administration of probiotics can downregulate both Th2-related allergy and Th1-related inflammatory symptoms is related to the action of probiotics to improve regulatory immune activity, as evidenced by the results of animal experiments[43]. Actually, low-grade inflammation was suggested as a key factor, not only in the pathogenesis of AD but also in IBS. Consistent with this, the administration of *B. bifidum* BGN4-containing probiotics improved both AD and irritable syndrome in 2 separate clinical trials, as described below. In double-blind, randomized, placebo-controlled human trials, infants who were perinatally administered a combination of *B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031 showed significantly lower prevalence and cumulative incidence of AD than a placebo group[26]. In a prospective, double-blind, randomized, placebo-controlled clinical study, IBS patients that received composite probiotics (*B. bifidum* BGN4, *B. lactis* AD011, *L. acidophilus* AD031, and *L. casei* IBS041) showed significant reductions in their IBS symptoms, including abdominal pain, after 8 weeks of treatment. This was observed particularly in the patients with mixed or diarrhea-predominant ailments[44]. However, an analysis of the function of the Tregs and the expression of Foxp3$^+$ does not have close clinical relevance when judging the efficacy of probiotics in AD patients, due to the various conflicting results pertaining to the relationship between the function of the regulatory cells and the occurrence of allergic symptoms. The suppressive function of the Tregs was diminished in infants with egg allergies[45]. Paradoxically, CD4+CD25+ Tregs expressing Foxp3$^+$ were increased in patients with AD compared to normal individuals[46]. Likewise, children with AD had significantly higher induced Foxp3$^+$ expression following stimulation with both house dust mites and ovalbumin (OVA) allergens compared to those without AD, which was suggested to reflect secondary compensatory mechanisms[47]. In addition, the administration of *L. acidophilus* LAVRI-A1 did not have significant effects on CD4+CD25+CTLA4+ cell numbers or Foxp3$^+$ expression in high-risk children[47].

In addition to probiotics, prebiotics have also shown some efficacy in ameliorating AD. When the effects of *L. salivarius* and fructooligosaccharide (synbiotic) with fructooligosaccharide alone (prebiotic) were compared in children with moderate to severe AD, the synbiotic combination was superior to the prebiotic alone for treating moderate to severe childhood AD[48].

Preschool children receiving synbiotics (*L. acidophilus* DDS-1, *B. lactis* UABLA-12 with fructooligosaccharide) showed a greater decrease in the mean SCORAD score and need for topical corticosteroids than children in the placebo group after 8 weeks. Interestingly, a flow cytometric analysis of lymphocyte subsets in the peripheral blood of patients in the probiotic group showed that the percentage of CD4 and the percentage and absolute count of CD25 decreased whereas the percentage and absolute count of CD8 increased[24].

While most of the clinical studies used live forms of probiotic bacteria, Hoang et al.[21] used the cell lysate of *L. rhamnosus,* reporting a substantial improvement in the quality of life, skin symptoms, and day and night-time irritation scores in children that received supplementation; however, this study was limited in its meaningfulness due to its open label, non-randomized clinical observation. The skin severity scores of AD decreased in the adult patients from baseline values at week 8 and week 12 when the subjects were given a diet containing heat-killed *L. paracasei*. However, the effect was largely limited because there was no significant difference between the *Lactobacillus* and placebo groups[20]. The administration of heat-killed *Lactobacillus* or administration onto established eczema may be a factor related to its weak effect observed in the study.

An effect of probiotics on the improvement of allergic diseases other than AD has also been reported. Daily supplementation with *L. gasseri* A5 for 8 weeks improved the clinical symptoms and immunoregulatory changes in school children suffering from asthma and AR[23]. The prevalence of "frequent wheezing" and "wheezing and/or noisy breathing apart from colds" was significantly lower in the synbiotic (*B. breve* M-16V and a galacto/fructooligosaccharide mixture) than in the placebo group, despite the fact that the total IgE levels did not differ between the groups[19].

The efficacy and actions of probiotics in animal studies

Recently, numerous publications have supported the effect of probiotics on the prevention and treatment of allergic diseases in animal studies. However, the suggested mechanisms related to their anti-allergic effects were variable. The action of probiotics to shift the immune system from the pathogenic Th2 response to a Th1/T regulatory response was demonstrated (Fig. 1). Oral treatment with the probiotic mixture VSL#3 was effective in redirecting allergen-specific Th2 polarized immune responses towards Th1/T regulatory responses. This treatment also offered protection against allergen-induced anaphylactic reactions in a murine model of food allergy[49]. The oral administration of *L. rhamnosus* GG in OVA-immunized rats induced OVA-specific hyporesponsiveness and reduced the OVA-induced proliferative response in mesenteric lymph nodes (MLNs) associated with $CD4^+CD25^+Foxp3^+$ T cell expansion and increased IL-10 and TGF-β secretion[51]. Exposure to the commensals and saprophytes in the absence of true danger signals from invasive pathogens and/or injured host cells were reported to induce the

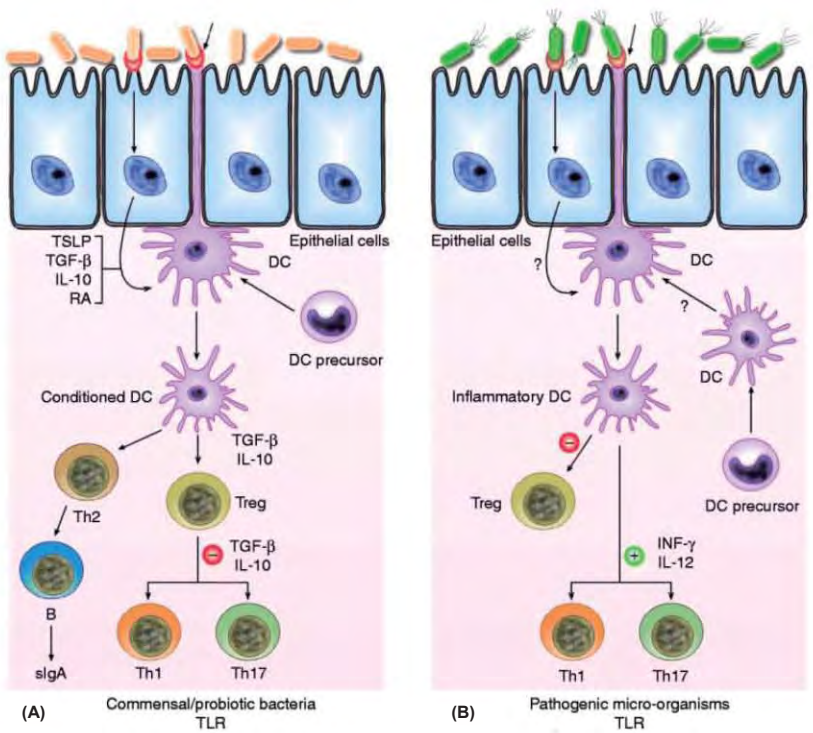

Fig. 1. Schematic view of the potential mechanism of action by which commensal bacteria and pathogenic bacteria interact with Toll-like receptors (TLRs) and elicit different immune responses. (A) Commensal and probiotic bacteria interact with intestinal epithelial-cell barrier and dendritic cells (DCs) resident in the intestine. Some cytokines, including interleukin (IL)-10, transforming growth factor beta (TGF-β) and thymic stromal lymphopoietin (TSLP), are expressed in intestinal epithelial cells, as a result of their interactions. Stimulation of cell TLR mediated by bacteria leads to up-regulation of TGF-β and IL-10, which in turn may limit the responsiveness of intestinal DCs resulting in the expansion and/or survival of T-cells with regulatory capacities, and limiting the ability of driving Th1, Th2 and Th17-cell responses. (B) Pathogenic bacteria have virulence factors that interact with intestinal epithelial-cell barrier and DCs resident in the intestine. Invasion of epithelium and direct interaction with DCs lead to activation of TLR and enhanced production of pro-inflammatory cytokines including interferon-gamma (IFN-γ) and IL-12, which are capable of driving Th1, Th2 and Th17 response. RA, retinoic acid; sIgA, secreted Ig A; Th, T helper cell; Treg, T regulatory cell (Reprinted from Gomez-Llorente C, Munoz S, Gil A. Proc Nutr Soc 2010; 69:381-9, with permission of Cambridge University Press)[50].

actions of regulatory network[41] such as Tregs and inducible Tregs (Th3, Tr1) as well as Foxp3$^+$. Foxp3$^+$ is crucial for both the differentiation of Tregs and the maintenance of their suppressive function[52]. The induction of bacterial strain-specific Foxp3$^+$ Tregs was evident in mice treated with *B. longum* AH1206 but not in mice treated with *L. salivarius* AH102, suggesting that the induction of Foxp3$^+$ Tregs was strain specific[53]. The regulatory network including, in addition to Tregs, DCs and the cytokines produced by these cells is essential in the development of tolerance. Kwon et al.[43] showed that IRT5, a probiotic mixture, exerted potent immunomodulatory effects by upregulating or enhancing the generation of Tregs by tolerogenic DCs in MLN. Moreover, the migration of CD4$^+$Foxp3$^+$ Tregs to sites of inflammation effectively suppressed disease progression. The enhanced therapeutic efficacy was associated with an increase in anti-inflammatory cytokines (IL-10 and TGF-β) as well as a decrease in pro-inflammatory cytokines.

The effect of *L. casei* in inhibiting allergic inflammation by acting at the effector phase of adaptive immune responses instead of at the initiation phase was also reported[54]. The suppressive effects of *L. gasseri* OLL2809 on inflammatory responses was associated with the suppression of CD4$^+$ T cell proliferation through a MyD88-dependent signaling pathway and by *L. gasseri* OLL2809 and its RNA[55].

The oral treatment of neonatal pigs with *L. lactis* significantly reduced the subsequent frequency of allergy to ovomucoid and was associated with lower IgG(1)/IgG(2) and IgE/IgG(2) ratios, indicating a Th1 bias and a reduced Th2 immune response[56]. Kim et al.[57] showed that *B. bifidum* BGN4 and *L. casei* appeared to be useful probiotic bacteria for the prevention of allergy, suggesting that these bacteria induce anti-allergenic processes through the induction of the Th1 response and the regulatory lymphocyte. Pochard et al.[58] demonstrated that *L. plantarum*, *L. lactis*, *L. casei*, and *L. rhamnosus* GG suppressed IL-4 and IL-5 (Th2 cytokines) and increased interferon-gamma (IFN-γ) and IL-12 (Th1 cytokines) in a dose-dependent manner, suggesting a more balanced Th1/Th2 response *in vitro* with human polymorphonuclear cells.

The observation that probiotics enhanced the production of Th1 and regulatory cytokines *in vitro* but slightly decreased the *ex vivo* production of IL-10, tumor necrosis factor-alpha, and IL-6 suggests that the effect of probiotics with regard to the immunomodulatory potential differed depending on the *in vitro* or *ex vivo* treatment[59]. Moreover, changes in the production of various cytokines and the activation of immunoregulatory cells as observed in animal studies have not been revealed in human studies.

Conclusions

Various meta-analysis and systematic review studies have shown positive effects of probiotics with regard to the prevention of AD, particularly in infants who were administered probiotics during the perinatal period. However, further studies regarding the optimal dose, effective probiotic strains, the timing and duration of supplementation, the additive/synergistic effects between probiotics and prebiotics, and patient populations that would most benefit from the use of probiotics need to be more thoroughly investigated. While there is substantial evidence for the amelioration of AD by probiotics from *in vitro* experiments and animal studies, the results from human clinical trials are more complicated. Thus elucidating the effects and mechanisms of action of probiotics is difficult due to the variation of results derived from human studies. Therefore, the mechanism involved in the preventive effect of probiotics in humans and the long-term effects of probiotics on the developing immune system remain to be proven.

Acknowledgment

This study was supported by the Small and Medium Business Administration (No. S1072365) and the Next-Generation BioGreen 21 Program (No. PJ008005), from the Rural Development Administration of the Republic of Korea.

References

1. Saeki H, Furue M, Furukawa F, Hide M, Ohtsuki M, Katayama I, et al. Guidelines for management of atopic dermatitis. J Dermatol 2009;36:563-77.
2. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006;368:733-43.
3. Williams H, Stewart A, von Mutius E, Cookson W, Anderson HR; International Study of Asthma and Allergies in Childhood (ISAAC) Phase One and Three Study Groups. Is eczema really on the increase worldwide? J Allergy Clin Immunol 2008;121:947-54.e15.
4. Williams H, Robertson C, Stewart A, Ait-Khaled N, Anabwani G, Anderson R, et al. Worldwide variations in the prevalence of symptoms of atopic eczema in the International Study of Asthma and Allergies in Childhood. J Allergy Clin Immunol 1999;103(1 Pt 1):125-38.
5. Batchelor JM, Grindlay DJ, Williams HC. What's new in atopic eczema? An analysis of systematic reviews published in 2008 and 2009. Clin Exp Dermatol 2010;35:823-7.
6. Ji GE. Probiotics in primary prevention of atopic dermatitis. Forum Nutr 2009;61:117-28.
7. Akdis CA, Blaser K, Akdis M. Apoptosis in tissue inflammation and

allergic disease. Curr Opin Immunol 2004;16:717-23.

8. World Health Organization, Food and Agriculture Organization of the United Nations [Internet]. Geneva: WHO/FAO; c2012 [2012 Jan 20]. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria: report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Available from: http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf.
9. Chapman CM, Gibson GR, Rowland I. Health benefits of probiotics: are mixtures more effective than single strains? Eur J Nutr 2011;50:1-17.
10. Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol 2001;108:516-20.
11. Gore C, Custovic A, Tannock GW, Munro K, Kerry G, Johnson K, et al. Treatment and secondary prevention effects of the probiotics Lactobacillus paracasei or Bifidobacterium lactis on early infant eczema: randomized controlled trial with follow-up until age 3 years. Clin Exp Allergy 2012;42:112-22.
12. Roessler A, Forssten SD, Glei M, Ouwehand AC, Jahreis G. The effect of probiotics on faecal microbiota and genotoxic activity of faecal water in patients with atopic dermatitis: a randomized, placebo-controlled study. Clin Nutr 2012;31:22-9.
13. Larsen N, Vogensen FK, Gobel R, Michaelsen KF, Abu Al-Soud W, Sorensen SJ, et al. Predominant genera of fecal microbiota in children with atopic dermatitis are not altered by intake of probiotic bacteria Lactobacillus acidophilus NCFM and Bifidobacterium animalis subsp. lactis Bi-07. FEMS Microbiol Ecol 2011;75:482-96.
14. Morisset M, Aubert-Jacquin C, Soulaines P, Moneret-Vautrin DA, Dupont C. A non-hydrolyzed, fermented milk formula reduces digestive and respiratory events in infants at high risk of allergy. Eur J Clin Nutr 2011;65:175-83.
15. Torii S, Torii A, Itoh K, Urisu A, Terada A, Fujisawa T, et al. Effects of oral administration of Lactobacillus acidophilus L-92 on the symptoms and serum markers of atopic dermatitis in children. Int Arch Allergy Immunol 2011;154:236-45.
16. Boyle RJ, Ismail IH, Kivivuori S, Licciardi PV, Robins-Browne RM, Mah LJ, et al. Lactobacillus GG treatment during pregnancy for the prevention of eczema: a randomized controlled trial. Allergy 2011;66:509-16.
17. Kukkonen AK, Savilahti EM, Haahtela T, Savilahti E, Kuitunen M. Ovalbumin-specific immunoglobulins A and G levels at age 2 years are associated with the occurrence of atopic disorders. Clin Exp Allergy 2011;41:1414-21.
18. Nermes M, Kantele JM, Atosuo TJ, Salminen S, Isolauri E. Interaction of orally administered Lactobacillus rhamnosus GG with skin and gut microbiota and humoral immunity in infants with atopic dermatitis. Clin Exp Allergy 2011;41:370-7.
19. van der Aa LB, van Aalderen WM, Heymans HS, Henk Sillevis Smitt J, Nauta AJ, Knippels LM, et al. Synbiotics prevent asthma-like symptoms in infants with atopic dermatitis. Allergy 2011;66:170-7.
20. Moroi M, Uchi S, Nakamura K, Sato S, Shimizu N, Fujii M, et al. Beneficial effect of a diet containing heat-killed Lactobacillus paracasei K71 on adult type atopic dermatitis. J Dermatol 2011;38:131-9.
21. Hoang BX, Shaw G, Pham P, Levine SA. Lactobacillus rhamnosus cell lysate in the management of resistant childhood atopic eczema. Inflamm Allergy Drug Targets 2010;9:192-6.
22. Dotterud CK, Storro O, Johnsen R, Oien T. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. Br J Dermatol 2010;163:616-23.
23. Chen YS, Jan RL, Lin YL, Chen HH, Wang JY. Randomized placebo-controlled trial of Lactobacillus on asthmatic children with allergic rhinitis. Pediatr Pulmonol 2010;45:1111-20.
24. Gerasimov SV, Vasjuta VV, Myhovych OO, Bondarchuk LI. Probiotic supplement reduces atopic dermatitis in preschool children: a randomized, double-blind, placebo-controlled, clinical trial. Am J Clin Dermatol 2010;11:351-61.
25. Rose MA, Stieglitz F, Koksal A, Schubert R, Schulze J, Zielen S. Efficacy of probiotic Lactobacillus GG on allergic sensitization and asthma in infants at risk. Clin Exp Allergy 2010;40:1398-405.
26. Kim JY, Kwon JH, Ahn SH, Lee SI, Han YS, Choi YO, et al. Effect of probiotic mix (Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial. Pediatr Allergy Immunol 2010;21(2 Pt 2):e386-93.
27. Woo SI, Kim JY, Lee YJ, Kim NS, Hahn YS. Effect of Lactobacillus sakei supplementation in children with atopic eczema-dermatitis syndrome. Ann Allergy Asthma Immunol 2010;104:343-8.
28. Niers L, Martin R, Rijkers G, Sengers F, Timmerman H, van Uden N, et al. The effects of selected probiotic strains on the development of eczema (the PandA study). Allergy 2009;64:1349-58.
29. Soh SE, Aw M, Gerez I, Chong YS, Rauff M, Ng YP, et al. Probiotic supplementation in the first 6 months of life in at risk Asian infants: effects on eczema and atopic sensitization at the age of 1 year. Clin Exp Allergy 2009;39:571-8.
30. Kuitunen M, Kukkonen K, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, et al. Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. J Allergy Clin Immunol 2009;123:335-41.
31. Hattori K, Yamamoto A, Sasai M, Taniuchi S, Kojima T, Kobayashi Y, et al. Effects of administration of bifidobacteria on fecal microflora and clinical symptoms in infants with atopic dermatitis. Arerugi 2003;52:20-30.
32. Savilahti E, Kuitunen M, Vaarala O. Pre and probiotics in the prevention and treatment of food allergy. Curr Opin Allergy Clin Immunol 2008;8:243-8.
33. Wickens K, Black PN, Stanley TV, Mitchell E, Fitzharris P, Tannock GW, et al. A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol 2008;122:788-94.
34. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 2001;357:1076-9.
35. Kopp MV, Hennemuth I, Heinzmann A, Urbanek R. Randomized, double-blind, placebo-controlled trial of probiotics for primary prevention: no clinical effects of Lactobacillus GG supplementation. Pediatrics 2008;121:e850-6.
36. Kim JY, Choi YO, Kwon JH, Ahn KM, Park MS, Ji GE. Clinical effects of probiotics are associated with increased transforming growth factor-β responses in infants with high-risk allergy. J Korean Soc Appl Biol Chem 2011;54:944-8.
37. Rautava S, Kalliomaki M, Isolauri E. Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic

disease in the infant. J Allergy Clin Immunol 2002;109:119-21.

38. Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH, et al. Effect of probiotic Lactobacillus strains in children with atopic dermatitis. J Allergy Clin Immunol 2003;111:389-95.
39. Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. Clin Exp Allergy 2000;30:1604-10.
40. Mastrandrea F, Coradduzza G, Serio G, Minardi A, Manelli M, Ardito S, et al. Probiotics reduce the CD34+ hemopoietic precursor cell increased traffic in allergic subjects. Eur Ann Allergy Clin Immunol 2004;36:118-22.
41. von Hertzen LC, Savolainen J, Hannuksela M, Klaukka T, Lauerma A, Mäkela MJ, et al. Scientific rationale for the Finnish Allergy Programme 2008-2018: emphasis on prevention and endorsing tolerance. Allergy 2009;64:678-701.
42. Ng SC, Kamm MA, Stagg AJ, Knight SC. Intestinal dendritic cells: their role in bacterial recognition, lymphocyte homing, and intestinal inflammation. Inflamm Bowel Dis 2010;16:1787-807.
43. Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, et al. Generation of regulatory dendritic cells and $CD4^+Foxp3^+$ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A 2010;107:2159-64.
44. Hong KS, Kang HW, Im JP, Ji GE, Kim SG, Jung HC, et al. Effect of probiotics on symptoms in Korean adults with irritable bowel syndrome. Gut Liver 2009;3:101-7.
45. Smith M, Tourigny MR, Noakes P, Thornton CA, Tulic MK, Prescott SL. Children with egg allergy have evidence of reduced neonatal CD4(+)CD25(+)CD127(lo/-) regulatory T cell function. J Allergy Clin Immunol 2008;121:1460-6, 1466.e1-7.
46. Ou LS, Goleva E, Hall C, Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. J Allergy Clin Immunol 2004;113:756-63..
47. Taylor AL, Hale J, Hales BJ, Dunstan JA, Thomas WR, Prescott SL. FOXP3 mRNA expression at 6 months of age is higher in infants who develop atopic dermatitis, but is not affected by giving probiotics from birth. Pediatr Allergy Immunol 2007;18:10-9.
48. Wu KG, Li TH, Peng HJ. Lactobacillus salivarius plus fructo-oligosaccharide is superior to fructo-oligosaccharide alone for treating children with moderate to severe atopic dermatitis: a double-blind, randomized, clinical trial of efficacy and safety. Br J Dermatol 2012;166:129-36.
49. Schiavi E, Barletta B, Butteroni C, Corinti S, Boirivant M, Di Felice G. Oral therapeutic administration of a probiotic mixture suppresses established Th2 responses and systemic anaphylaxis in a murine model of food allergy. Allergy 2011;66:499-508.
50. Gomez-Llorente C, Munoz S, Gil A. Role of Toll-like receptors in the development of immunotolerance mediated by probiotics. Proc Nutr Soc 2010;69:381-9.
51. Finamore A, Roselli M, Britti MS, Merendino N, Mengheri E. Lactobacillus rhamnosus GG and Bifidobacterium animalis MB5 induce intestinal but not systemic antigen-specific hyporesponsiveness in ovalbumin-immunized rats. J Nutr 2012;142:375-81.
52. Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006;24:209-26.
53. Lyons A, O'Mahony D, O'Brien F, MacSharry J, Sheil B, Ceddia M, et al. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. Clin Exp Allergy 2010;40:811-9.
54. Schiffer C, Lalanne AI, Cassard L, Mancardi DA, Malbec O, Bruhns P, et al. A strain of Lactobacillus casei inhibits the effector phase of immune inflammation. J Immunol 2011;187:2646-55.
55. Yoshida A, Yamada K, Yamazaki Y, Sashihara T, Ikegami S, Shimizu M, et al. Lactobacillus gasseri OLL2809 and its RNA suppress proliferation of CD4(+) T cells through a MyD88-dependent signalling pathway. Immunology 2011;133:442-51.
56. Rupa P, Schmied J, Wilkie BN. Prophylaxis of experimentally induced ovomucoid allergy in neonatal pigs using Lactococcus lactis. Vet Immunol Immunopathol 2011;140:23-9.
57. Kim H, Kwack K, Kim DY, Ji GE. Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model. FEMS Immunol Med Microbiol 2005;45:259-67.
58. Pochard P, Gosset P, Grangette C, Andre C, Tonnel AB, Pestel J, et al. Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. J Allergy Clin Immunol 2002;110:617-23.
59. Flinterman AE, Knol EF, van Ieperen-van Dijk AG, Timmerman HM, Knulst AC, Bruijnzeel-Koomen CA, et al. Probiotics have a different immunomodulatory potential in vitro versus ex vivo upon oral administration in children with food allergy. Int Arch Allergy Immunol 2007;143:237-44.

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GENOME ANNOUNCEMENT

Complete Genome Sequence of the Probiotic Bacterium *Bifidobacterium bifidum* Strain BGN4

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***Bifidobacterium bifidum*, a common endosymbiotic inhabitant of the human gut, is considered a prominent probiotic microorganism that may promote health. We completely decrypted the 2.2-Mb genome sequence of *B. bifidum* BGN4, a strain that had been isolated from the fecal sample of a healthy breast-fed infant, and annotated 1,835 coding sequences.**

Bifidobacterium bifidum BGN4, a β-glucosidase-negative strain that was isolated from the fecal sample of a healthy, breast-fed infant, first drew our special attention because the β-glucosidase activity in the intestine can produce carcinogenic or mutagenic aglycones from various glycosides such as rutin, guercitrin, robinin, and cycasine (3). As probiotics responsible for intestinal healthiness, there are several lines of evidence from *in vitro* and *in vivo* experiments supporting the notion that bifidobacteria can modulate the host immune system and inhibit pathogen infection (11). In particular, the anticarcinogenic polysaccharide isolated from the cytosolic fraction of BGN4 inhibits the growth of some cancer cell lines (19) and it was also reported to have a potent adhering activity with respect to Caco-2 cells and to be able to alleviate allergic reactions elicited by ovalbumin in a mouse model (9, 10).

The genome sequence was determined by the use of a Roche GS FLX system (NICEM, Republic of Korea). A total of 209,020 reads totaling up to 23.18× coverage were assembled into 27 contigs using GS Assembler. Gap closing was performed by multiplex PCR and primer walking on the amplified products by the standard Sanger sequencing. Sequence manipulation, primer design, and manual validation were performed using Phred/Phrap/CONSED (6). Protein-coding genes were predicted by the combination of CRITICA (2) and GLIMMER (4). tRNAs and rRNAs were identified by tRNAScan-SE (15) and BLAST (1), respectively. All predicted genes were annotated by AutoFACT (12), with additional searches performed using the TIGRFAMs database (18) and protein sequences from the genomes of *B. longum* species (13, 17) and *B. adolescentis* ATCC 15703.

The complete sequence consists of a 2,223,664-bp circular chromosome (62.65% G+C) with no plasmid. We compiled 1,835 coding sequences (CDSs), 7 pseudogenes, 3 rRNA operons, and 52 tRNAs from the nucleotide sequence. A total of 1,373 CDSs were assigned predicted functions, while the rest was designated conserved hypothetical proteins or hypothetical proteins. The genome contains 27 insertion sequence elements or transposons and 20 kinds of aminoacyl-tRNA synthetase genes. In particular, a BGN4-specific 52-kb segment (bp 1392576 to 1445526) encoding two mobilization proteins (MobC [BBB_1196] and MobA [BBB_1198]), 16 functional proteins, and 28 hypothetical proteins was identified by genomewise comparison with *B. bifidum* PRL2010, which might have been acquired by horizontal gene transfer.

The genome sequence analysis helps elucidate the phenotypic features of BGN4, including its probiotic effects. For example, the gene encoding glutamine fructose-6-phosphate amidotransferase (GlmS [BBB_0791]) that is involved in *N*-acetylglucosamine biosynthesis is interrupted by a stop codon to make it a pseudogene, which might be responsible for the *N*-acetylglucosamine auxotrophy of *B. bifidum* (5). Moreover, the presence of a homolog (BBB_0596) of the bifidobacterial outer protein (BopA) (7) suggests its high capacity for adhesion to the Caco-2 cell line. Deconjugation of bile salts and the reduction of serum cholesterol levels are closely related (14, 16), and BBB_0854, homologous to bile salt hydrolase (EC 3.5.1.24), may contribute to bile salt tolerance.

Nucleotide sequence accession number. Genome sequence information was registered in GenBank under accession number CP001361. The sequence and annotation are also available from the Genome Encyclopedia of Microbes (GEM; https://www.gem.re.kr) (8).

ACKNOWLEDGMENTS

We are grateful for the financial assistance from the 21C Frontier Microbial Genomics and Applications Center program of the Ministry of Education, Science and Technology, National Research Foundation of Korea (2011-0017670) and the KRIBB Research Initiative Program.

REFERENCES

1. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
2. **Badger JH, Olsen GJ.** 1999. CRITICA: coding region identification tool invoking comparative analysis. Mol. Biol. Evol. **16**:512–524.
3. **Choi YJ, et al.** 1996. Growth and β-glucosidase activity of *Bifidobacterium*. J. Microbiol. Biotechnol. **6**:255–259.
4. **Delcher AL, Harmon D, Kasif S, White O, Salzberg SL.** 1999. Improved

Received 2 June 2012 **Accepted** 19 June 2012

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doi:10.1128/JB.00988-12

microbial gene identification with GLIMMER. Nucleic Acids Res. **27**: 4636–4641.
5. **Foley S, et al.** 2008. Characterisation of glutamine fructose-6-phosphate amidotransferase (EC 2.6.1.16) and *N*-acetylglucosamine metabolism in *Bifidobacterium.* Arch. Microbiol. **189**:157–167.
6. **Gordon D, Abajian C, Green P.** 1998. Consed: a graphical tool for sequence finishing. Genome Res. **8**:195–202.
7. **Guglielmetti S, et al.** 2008. Implication of an outer surface lipoprotein in adhesion of *Bifidobacterium bifidum* to Caco-2 cells. Appl. Environ. Microbiol. **74**:4695–4702.
8. **Jeong H, Yoon SH, Yu DS, Oh TK, Kim JF.** 2008. Recent progress of microbial genome projects in Korea. Biotechnol. J. **3**:601–611.
9. **Kim H, Kwack K, Kim DY, Ji GE.** 2005. Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model. FEMS Immunol. Med. Microbiol. **45**:259–267.
10. **Kim IH, Park MS, Ji GE.** 2003. Characterization of adhesion of *bifidobacterium* sp. BGN4 to human enterocyte-like caco-2 cells. J. Microbiol. Biotechnol. **13**:276–281.
11. **Kim JF, et al.** 2009. Genome sequence of the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* AD011. J. Bacteriol. **191**:678–679.
12. **Koski LB, Gray MW, Lang BF, Burger G.** 2005. AutoFACT: an automatic functional annotation and classification tool. BMC Bioinformatics **6**:151.
13. **Lee JH, et al.** 2008. Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. BMC Genomics **9**:247.
14. **Liong MT, Shah NP.** 2005. Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. Int. Dairy J. **15**:391–398.
15. **Lowe TM, Eddy SR.** 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. **25**: 955–964.
16. **Noriega L, Cuevas I, Margolles A, de los Reyes-Gavilán CG.** 2006. Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile. Int. Dairy J. **16**:850–855.
17. **Schell MA, et al.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. U. S. A. **99**:14422–14427.
18. **Selengut JD, et al.** 2007. TIGRFAMs and Genome Properties: tools for the assignment of molecular function and biological process in prokaryotic genomes. Nucleic Acids Res. **35**:D260–D264.
19. **You HJ, Oh DK, Ji GE.** 2004. Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from *Bifidobacterium bifidum* BGN4. FEMS Microbiol. Lett. **240**:131–136.

International Journal of
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Safety Evaluations of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI

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Received: 6 April 2018; Accepted: 2 May 2018; Published: 9 May 2018

Abstract: Over the past decade, a variety of lactic acid bacteria have been commercially available to and steadily used by consumers. However, recent studies have shown that some lactic acid bacteria produce toxic substances and display properties of virulence. To establish safety guidelines for lactic acid bacteria, the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) has suggested that lactic acid bacteria be characterized and proven safe for consumers' health via multiple experiments (e.g., antibiotic resistance, metabolic activity, toxin production, hemolytic activity, infectivity in immune-compromised animal species, human side effects, and adverse-outcome analyses). Among the lactic acid bacteria, *Bifidobacterium* and *Lactobacillus* species are probiotic strains that are most commonly commercially produced and actively studied. *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI have been used in global functional food markets (e.g., China, Germany, Jordan, Korea, Lithuania, New Zealand, Poland, Singapore, Thailand, Turkey, and Vietnam) as nutraceutical ingredients for decades, without any adverse events. However, given that the safety of some newly screened probiotic species has recently been debated, it is crucial that the consumer safety of each commercially utilized strain be confirmed. Accordingly, this paper details a safety assessment of *B. bifidum* BGN4 and *B. longum* BORI via the assessment of ammonia production, hemolysis of blood cells, biogenic amine production, antimicrobial susceptibility pattern, antibiotic resistance gene transferability, PCR data on antibiotic resistance genes, mucin degradation, genome stability, and possession of virulence factors. These probiotic strains showed neither hemolytic activity nor mucin degradation activity, and they did not produce ammonia or biogenic amines (i.e., cadaverine, histamine or tyramine). *B. bifidum* BGN4 and *B. longum* BORI produced a small amount of putrescine, commonly found in living cells, at levels similar to or lower than that found in other foods (e.g., spinach, ketchup, green pea, sauerkraut, and sausage). *B. bifidum* BGN4 showed higher resistance to gentamicin than the European Food Safety Authority (EFSA) cut-off. However, this paper shows the gentamicin resistance of *B. bifidum* BGN4 was not transferred via conjugation with *L. acidophilus* ATCC 4356, the latter of which is highly susceptible to gentamicin. The entire genomic sequence of *B. bifidum* BGN4 has been published in GenBank (accession no.: CP001361.1), documenting the lack of retention of plasmids capable of transferring an antibiotic-resistant gene. Moreover, there was little genetic mutation between the first and 25th generations of *B. bifidum* BGN4. Tetracycline-resistant genes are prevalent among *B. longum* strains; *B. longum* BORI has a *tet*(W) gene on its chromosome DNA and

has also shown resistance to tetracycline. However, this research shows that its tetracycline resistance was not transferred via conjugation with *L. fermentum* AGBG1, the latter of which is highly sensitive to tetracycline. These findings support the continuous use of *B. bifidum* BGN4 and *B. longum* BORI as probiotics, both of which have been reported as safe by several clinical studies, and have been used in food supplements for many years.

Keywords: probiotics; safety; antibiotics resistance; functional foods; nutraceuticals

1. Introduction

Since "probiotics" first emerged in the 1960s [1], the term has been defined by various scholars and groups. In recent years, probiotics have been clearly defined by several regulatory organizations [2]. According to the FAO/WHO, probiotics can be defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" [3]. Other experts similarly define probiotics as "live microorganisms which, when ingested or locally applied in sufficient numbers, provide the consumer with one or more proven health benefits" [4]. Edible microorganisms regarded as probiotic bacteria are derived from various strains, species, and genera, which have been studied with regard to various human health benefits [5]. A variety of microorganisms, including *Bacillus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., and *Propionibacterium* spp., are regarded as probiotics, and are known to be involved in the vitamin biosynthesis of the host's nutrition metabolism and physiological function via immune-mediated effects [6,7]. Of these probiotic microorganisms, *Lactobacillus* spp. and *Bifidobacterium* spp. have been utilized globally in fermented food products and commercially-produced food supplements [8]. As of July 2010, the genomic sequences of approximately 11 *Bifidobacterium* and 21 *Lactobacillus* species have been completely analyzed, whose microbial genomic sequences offer exact evidence of the probiotics' genera and species [9]. Some experts have found that consumer demand for food or food supplements containing lactic acid bacteria have led to the exponential growth of healthy trends in the global food market [10]. However, this phenomenon cannot disregard microbial safety standards or allow lactic acid bacteria to be used indiscriminately without scientific research or safety verification [3,11]. Also, a probiotic safety assessment should consider the probiotic's physiological characteristics, treatment method (e.g., oral administration, skin spray, gel, capsule, etc.), exposure dosage, consumers' health, and the physiological functions required for effective probiotic performance [12].

In 2002, the FAO created four basic guidelines for food industry probiotic application, because a variety of commercially-available microorganisms had been sold to consumers as probiotics without clear labeling standards. The FAO guidelines summarized by Huys et al. [13] are as follows: (i) "the assessment of strain identity (i.e., genus, species, and strain level); (ii) in vitro tests to screen potential probiotic strains (e.g., resistance to gastric acidity, bile acid, and digestive enzymes, antimicrobial activity against potentially pathogenic bacteria, etc.); (iii) assessment of safety: requirement of proof that a probiotic strain is safe and without contamination in its delivery form; and (iv) in vivo studies for the substantiation of the health effects in the target host". In addition, the FAO recommended that various tests (e.g., analysis of antibiotic resistance, metabolic activity, toxin production, hemolytic activity, infectivity in immune-compromised animal models, human side effects, and adverse outcomes in consumers) be conducted with the probiotic microorganisms to demonstrate their safety to hosts and elaborate on section three of the aforementioned guidelines [3]. However, these safety assessment items are recommendations rather than legal requirements. Various research groups have evaluated the safety of probiotic bacteria according to their cell types and microbial functionalities by incorporating additional experimental methods [14–16]. In 2002, the European Union Scientific Committee on Animal Nutrition issued guidelines for the safety assessment and regulation of edible microorganisms utilized in food and animal feeds. The corresponding "qualified presumption of safety (QPS)" guidelines

from 2016 are as follows [11]: (1) definition of the taxonomy of the microbe; (2) collection of sufficient information providing the basis for QPS status, including any scientific literature, history of use, industrial applications, ecological data, and human intervention data; (3) exclusion of pathogenicity; and (4) definition of the end use. Based on this guideline, QPS status may be granted to probiotic cells in European Union food markets if there are no safety problems with a particular taxon or if the safety problem is alleviated. It is commonly agreed that microbial safety should demonstrate the (i) species characteristics with genetic information, (ii) phenotypic evidence, (iii) isolation history, (iv) absence/presence of antibiotic-resistant properties, and (v) potential virulence and/or pathogenic factors [17].

One of the greatest safety concerns for commercially-produced lactic acid bacteria is that some of the microorganisms supplied in the form of diets may act as the donor of antibiotic-resistant plasmids to intestinal pathogens [18,19]. Several reports have found that in the presence of antibiotic treatment, some strains survive in the human gastrointestinal tract due to the transferred resistance of plasmids [20–22]. A variety of microbial genes can be transferred to enteric bacteria in the intestine via plasmids, resulting in the spread of antibiotic-resistance [23]. Therefore, ensuring the safety of a probiotic strain is necessary prior to the mass production of lactic acid bacteria for commercial purposes.

Although some *Bifidobacterium* and *Lactobacillus* spp. have shown promise in in vivo and in vitro studies, there is a lack of clear clinical evidence to support the health benefits of these microorganisms [24]. Therefore, many groups and researchers are trying to prove the efficacy of lactic acid bacteria through clinical experimentation. *B. bifidum* BGN4 and *B. longum* BORI were isolated from the feces of healthy breast-feeding infants, and have been commercially used as food ingredients since 2000 [25–29]. Some bifidobacteria strains, including *B. bifidum* and *B. longum*, are registered as functional ingredient, Probiotics (II.2.51) in Health Functional Food Code of Korea [30]. Over the years, many studies have revealed the functionalities of *B. bifidum* BGN4 [28], and its complete genomic sequence was reported to GenBank [31]. *B. longum* BORI, also isolated from a healthy breast-fed infant and deposited in KCCM (Korean Culture Center of Microorganisms, 14092), was proven to statistically shorten the duration of diarrhea in a clinical study of infants infected with rotavirus [25]. Both probiotic strains have been proven to effectively form healthy intestinal microflora without any adverse effects. However, further systematic research should be conducted to prove their safety for academic and commercial applications. The aim of this study was to validate the safety of *B. bifidum* BGN4 and *B. longum* BORI by conducting FAO/WHO recommended experiments and other published safety research.

2. Results and Discussion

2.1. Ammonia Production

Intestinal bacteria can degrade various nitrogen sources (e.g., proteins, peptides, and amino acids) present in the feces of the intestinal track [32]. These naturally-occurring microbiota and artificially-administered flora have the potential to produce various toxic substances during the deamination stage via nitrogen derivatives. Multiple potentially toxic products (i.e., phenol, ammonia, and indole [33], are possible products of the proteolytic process, especially in the large intestine. Thus, bacterial ammonia production is highly relevant to human intestinal health, and a necessary component of the evaluation to demonstrate the safety of commercial probiotics. Moreover, recent studies have also shown that ammonia produced by gut microorganisms can affect the liver and act as a cofactor in chronic liver damage. Vince and Burridge [34] reported that considerable amounts of ammonia were generated by the Gram-negative anaerobes, *Clostridia* (including *Clostridium perfringens*), *Enterobacter*, and *Bacillus* spp. Some strains of streptococci, micrococci, and Gram-positive non-spore forming anaerobes produced moderate concentrations of ammonia. By contrast, Gram-positive aerobic rods, in particular *Lactobacilli*, produced very little ammonia.

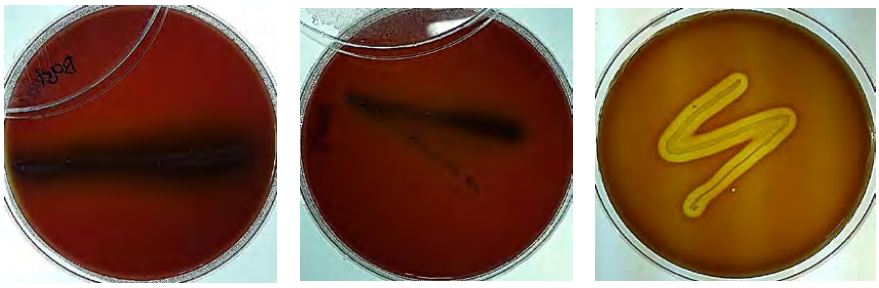
The ammonia production of *B. bifidum* BGN4 and *B. longum* BORI were assessed to verify the safety of these probiotics. In this study, *B. bifidum* BGN4, *B. longum* BORI, and other probiotic strains did not produce ammonia. By contrast, *Bacteroides* spp., *Clostridium perfringens,* and *Enterobacter* spp., which are known harmful bacteria and used as positive controls in this study, produced 12.9 ± 1.3 to 161.0 ± 6.6 μg/mL of ammonia (Table 1). This test included three replications, and the values presented are the means ± the standard deviations. This study found no indication of the production of ammonia by *B. bifidum* BGN4 and *B. longum* BORI.

Table 1. Mean value and standard deviation of ammonia level variables of *B. bifidum* BGN4, *B. longum* BORI, and other commercial microorganisms (*n* = 3).

Strain	Ammonia (μg/mL)
Bifidobacterium bifidum BGN4	negative
Bifidobacterium longum BORI	negative
Bifidobacterium breve ATCC 15701	negative
Lactobacillus plantarum KFRI 708	negative
Bacteroides fragilis ATCC 25285	14.7 ± 1.5
Bacteroides thetaiotaomicron ATCC 29741	23.3 ± 3.0
Clostridium perfringens ATCC 13124	23.5 ± 1.6
Enterobacter cloacae ATCC 13047	161.0 ± 6.6
Enterobacter faecalis ATCC 19433	12.9 ± 1.3

2.2. Hemolytic Property Test

The 2002 FAO/WHO Guidelines on Probiotics Safety Considerations clearly states that "if the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required" [3]. Microbial hemolysis properties are a common concern for pathogenic bacteria (e.g., enterococci, and streptococci) because of the potential for anemia and edema in the host. Although *Bifidobacterium* spp. are normal, naturally-occurring intestinal microbiota that have been widely included in functional foods and utilized by nutraceutical industries, they can potentially behave as opportunistic pathogenic microorganisms similar to common commensal microorganisms. Therefore, hemolysis assay tests should be conducted on potential probiotic bacteria. Visualizing the physical changes caused by hemolytic activity by culturing the microorganisms on a medium containing animal or human blood is a commonly used tool to evaluate the hemolytic properties of pathogens. In this study, the potential hemolytic activity of *B. bifidum* BGN4 and *B. longum* BORI were assessed using the blood agar plating method. *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 (positive control) showed β-hemolysis colorless zones around the cell colonies, whereas *B. bifidum* BGN4 and *B. longum* BORI showed no hemolysis and no change of color in the periphery of the colonies (Figure 1).



(**a**) (**b**) (**c**)

Figure 1. *B. bifidum* BGN4 ((**a**); back light) and *B. longum* BORI ((**b**); back light) growth with no blood cell lysis. Complete lysis of red blood cells was observed, with clear zones around the *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 colonies ((**c**); positive control, back light).

2.3. Evaluation of Biogenic Amine Production

Biogenic amines (e.g., cadaverine, histamine, tyramine, and putrescine) have hydrophobic skeletons and naturally-occurring organic polycation molecules derived from the amino acids in animals and humans [35]. These molecules are involved in multiple metabolic and intracellular activities of mammals (e.g., synaptic transmission, blood pressure control, allergic response, and cellular growth control). Traditionally, a variety of probiotic bacteria have been artificially integrated into fermented foods, due to their beneficial effects and flavor-enhancing properties [36]. Their biogenic amine levels have been regarded as an indicator of microbial activity and food freshness due to the fact that biogenic amines are generated via microbial metabolic activities (i.e., decarboxylation and the transamination of protein molecules) [37]. While biogenic amines are commonly found in fresh meat, vegetables, and cheese, ingestion of large amounts of biogenic amines may cause symptoms in humans and animals that are similar to severe allergic reactions [38]. One of the most common issues in the probiotics field in recent years has been whether probiotics contribute to the production of biogenic amines, and how they contribute to the production of biogenic amines [15]. Complex biogenic amines (i.e., polyamines having more than one amino group) were initially thought to be naturally present in a variety of fresh foods, but recent studies have shown that these chemicals can accumulate as a result of microbial activity. Some edible microorganisms and probiotic strains were reported to produce biogenic amines [39–41]. Therefore, the aim of this study was to examine the biogenic amine production of *B. bifidum* BGN4 and *B. longum* BORI as a component of an overall probiotic safety evaluation. The biogenic amine content of the bifidobacteria is featured in Table 2.

Table 2. Biogenic amine levels of *B. bifidum* BGN4 and *B. longum* BORI.

Strains	Cadaverine (μg/mL)	Histamine (μg/mL)	Putrescine (μg/mL)	Tyramine (μg/mL)
B. bifidum BGN4	N/D [1]	N/D [1]	24.23	N/D [1]
B. longum BORI	N/D [1]	N/D [1]	16.58	N/D [1]

[1] N/D; not detected.

The biogenic amine content of these strains was derived by subtracting the background content of the biogenic amines in each medium. *B. bifidum* BGN4 and *B. longum* BORI did not produce cadaverine, histamine, or tyramine; however, they produced 24.23 and 16.58 μg/mL of putrescine, respectively. The levels produced were not of concern. Putrescine is a natural substance present in various foods [42–44]. Putrescine, also naturally found in small amounts in living cells, is formed by the decarboxylation of ornithine and arginine. It is also a metabolite produced by various edible probiotic cells. Putrescine is also a precursor of spermidine and spermine. The polyamines putrescine, spermidine, spermine, and cadaverine are essential components of living cells, and play an important role in the formation of nucleic acid, protein synthesis, and membrane stability. Of the various biogenic amines detected in a variety of fruits, juices, and vegetables, putrescine was the most common. Kalač [42] reported that putrescine was commonly found in frozen spinach puree (average 12.9 mg/kg), ketchup (average 52.5 mg/kg), concentrated tomato paste (average 25.9 mg/kg), and frozen green pea (average 46.3 mg/kg). The putrescine content of fermented foods and beverages [43] was found to be 9 mg/kg (3–25 mg/kg, n = 28) in sherry, 154 mg/kg (6–550 mg/kg, n = 8) in sauerkraut, 19 mg/kg (1–71 mg/kg, n = 8) in Dutch cheese, and 52 mg/kg (1–190 mg/kg, n = 14) in fermented sausage. Furthermore, the putrescine found in the traditional cheeses made from ewe's whole milk in Sardinia, Italy, increased to 1658 mg/L during ripening [44]. *Bifidobacterium* spps. (i.e., *Bifidobacterium* CCDM 94, *B. adolescentis* CCDM 223, *B. animalis* ssp. *lactis* CCDM 239, 240, 241, and 374, *B. bifidum* CCDM 559, and *B. longum* CCDM 569) are known to produce cadaverine, putrescine, tyramine, and spermidine [41]. According to Pollark et al. [45], putrescine is contained in human breast milk (0~3804 nmol/L) and commercial formula milk (0~1057 ± 25 nmol/L).

Therefore, it matters how much putrescine occurs naturally. Some researchers theorize that putrescine in food is likely to show synergistic effects on histamine toxicity. However, such synergy has not been proven or reported with experimental data, as far as we know. Moreover, the European Food Safety Authority (EFSA) [46] also identified a lack of research to identify the exact levels of putrescine required to increase the side effects of histamine. *B. bifidum* BGN4 and *B. longum* BORI did not produce any cadaverine, histamine, or tyramine during the fermentation process. *B. bifidum* BGN4 and *B. longum* BORI produced low levels of putrescine, which was also found in both media (i.e., whole milk medium = 24.43 μg/mL, *B. bifidum* BGN4 culturing medium (whole milk) = 48.67 μg/mL, MRS medium = 26.60 μg/mL, *B. longum* BORI culturing medium (MRS) = 43.17 μg/mL). The human oxidation system of mono-amine and diamine oxidase includes small amounts of biogenic amines that are usually metabolized and harmless, because humans and animals have the ability to decompose them in vivo.

2.4. Antimicrobial Susceptibility and Antibiotic Resistance Transferability

2.4.1. Antibiotic Susceptibility

Various lactic acid bacteria research groups have warned that some lactic acid bacteria consumed as food or feed may have antibiotic-resistant properties. Since this resistance capability could be transferred to other pathogens via plasmids, the assessment of antibiotic resistance is an important criterion for evaluating the safety of strains used in food and feed [47]. Moreover, the acquired transferable genes have been characterized in bifidobacteria and lactobacilli [48]. In order to distinguish antibiotic-resistant from antibiotic-susceptible microorganisms, the EFSA has established microbiological cut-off values for the antibiotic-resistance of microorganisms used as food and/or feed additives. These microbiological cut-off values were determined based on the distribution of the chosen antimicrobials' minimum inhibitory concentrations (MICs) in cell populations belonging to a single taxonomical unit [49].

All *Bifidobacterium* spp. in this study were susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, penicillin G, rifampicin, and vancomycin (MIC ranging from 0.01 to 4 μg/mL) and generally resistant to aminoglycoside antibiotics, such as gentamicin, kanamycin, neomycin, and streptomycin (MIC ranging from >32 μg/mL, Table 3). The MIC values of *B. bifidum* BGN4 and *B. longum* BORI, with the exception of gentamicin and tetracycline, were equal to or lower than the established EFSA cut-off values suggested by the EFSA's Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) [49]. The susceptibility tendencies of *B. bifidum* BGN4 and *B. longum* BORI were similar to other studies [50–52], with the exception of high MIC to tetracycline in *B. longum* BORI. Penicillin G, ampicillin, vancomycin, gentamicin, erythromycin, trimethoprim–sulfamethoxazole, and metronidazole are known as frequently used antibiotics in pediatric patients [53]. *B. bifidum* BGN4 and *B. longum* BORI are resistant to trimethoprim–sulfamethoxazole but six of ten *Bifidobacterium* spp. strains also showed MIC values over 128 μg/mL in this research (Table 3).

Mättö et al. [54] reported *Bifidobacterium* strains displayed generally high MICs for streptomycin and gentamicin, and suggested their resistances were intrinsic. Ammor et al. [48] isolated probiotic bacteria from 21 food samples, such as yogurt, yogurt-type fermented milk, and pharmaceutical products, and found 22 strains of *Bifidobacterium* spp. In their study, Bifidobacteria were resistant to aminoglycoside (MIC_{90} ranges from 64 to 1000 μg/mL) and strongly resistant to kanamycin (MIC_{90} = 1000 μg/mL). They also demonstrated that some MIC ranges did not overlap, implying that the antibiotics related to these MIC ranges are usable as ingredients in selective media. They suggested the selective range of gentamicin was from 32 to 64 μg/mL and kanamycin was 64 to 500 μg/mL for *Bifidobacterium*. Therefore, gentamicin containing medium [55] and mupirocin containing medium [56,57] have been used for the selection and enumeration of *Bifidobacterium*. Accordingly, this resistance could be considered as intrinsic. Antibiotic resistance transferability studies were conducted to confirm the nature of this resistance.

Table 3. Antimicrobial susceptibility (MIC values) of *B. bifidum* BGN4 and *B. longum* BORI and other *Bifidobacterium* spp.

Antibiotics	EFSA Cut-Off of *Bifidobacterium* spp.	*B. longum* ATCC 15707	*B. longum* BB536	*B. longum* KCCM 91563	*B. longum* BORI	*B. infantis* ATCC 15697	*B. lactis* BB-12	*B. bifidum* BGN4	*B. bifidum* KCTC 3440	*B. adolescentis* ATCC 15703	*B. breve* M-16V	*E. faecalis* ATCC 29212
Penicillin G		0.25	0.125	0.5	1	0.125	0.125	0.063	0.063	0.25	0.25	0.5
Carbenicillin disodium salt		2	2	4	8	0.5	2	0.5	0.5	4	4	8
Methicillin		8	4	16	16	1	2	1	0.5	8	8	16
Ampicillin sodium salt	2	0.5	0.25	1	0.5	0.125	0.125	0.063	0.063	0.25	0.25	0.25
Dicloxacillin sodium salt hydrate		4	4	8	8	0.5	4	0.5	1	256	8	4
Gentamicin sulfate	64	32	64	32	32	16	128	128	256	128	128	256
Streptomycin sulfate salt	128	32	128	64	64	>256	128	64	32	128	256	>256
Kanamycin sulfate	N/R [1]	512	1024	1024	512	32	1024	1024	1024	1024	1024	256
Neomycin sulfate		1024	512	512	512	64	512	1024	512	512	1024	1024
Cephalothin sodium salt		8	4	16	32	4	8	4	2	16	16	16
Tetracycline	8	1	1	1	64	2	16	1	1	8	16	32
Polymyxin B sulfate salt		256	32	256	256	128	256	512	512	512	1024	>1024
Erythromycin	1	0.125	0.5	0.5	0.5	0.125	0.125	0.125	0.125	0.125	0.125	8
Metronidazole		8	8	>256	>256	8	4	4	64	>256	8	>256
Vancomycin hydrochloride	2	0.5	<0.25	<0.25	1	0.5	0.5	1	2	0.5	0.5	2
Chloramphenicol	4	2	2	2	4	2	2	2	2	2	2	8
Rifampicin		<0.125	<0.125	<0.125	0.25	<0.125	2	0.5	0.25	0.5	1	0.5
Clindamycin hydrochloride	1	<0.032	0.063	0.063	0.125	0.25	<0.032	0.063	0.063	<0.032	<0.032	>16
Phosphomycin disodium salt		128	256	256	256	16	64	128	256	64	32	32
Mupirocin		>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	64
Trimethoprim–Sulfamethoxazole		128	256	128	256	256	1	128	64	1	2	32

[1] N/R denotes not required.

2.4.2. Antibiotic Resistance Transferability

Since *B. bifidum* BGN4 and *B. longum* BORI showed high antibiotic resistance to gentamicin and/or tetracycline in these antimicrobial susceptibility tests, tetracycline resistance transferability tests were conducted using *L. fermentum* AGBG1, a recipient strain that is highly susceptible to tetracycline. In order to test the transferability of gentamicin resistance of *B. bifidum* BGN4 and *B. longum* BORI, *L. acidophilus* ATCC 4356 was used as a recipient strain, due to its high gentamicin sensitivity. The conjugation results are shown in Table 4.

Table 4. Transferability of tetracycline resistance from donors (*B. longum* BORI and *B. bifidum* BGN4) to recipients (*L. fermentum* AGBG1 and *L. acidophilus* ATCC 4356) (cfu/mL).

Antibiotics	**AGBG1 (Aerobic)**	**AGBG1 + BORI**		**BORI (Anaerobic)**
		Aerobic	**Anaerobic**	
None [1]	4.38×10^8	3.38×10^8	2.27×10^8	4.56×10^8
T8 [2]	0	0	4.44×10^6	7.11×10^7
Antibiotics	ATCC 4356 (Aerobic)	ATCC 4356 + BORI Aerobic	Anaerobic	BORI (Anaerobic)
None [1]	3.65×10^8	1.67×10^8	2.34×10^8	3.14×10^8
G64 [3]	0	0	2.78×10^6	1.46×10^8
Antibiotics	ATCC 4356 (Aerobic)	ATCC 4356 + BGN4 Aerobic	Anaerobic	BGN4 (Anaerobic)
None [1]	3.65×10^8	3.29×10^8	2.54×10^8	3.86×10^8
G64 [3]	0	0	4.64×10^6	1.43×10^8

[1] No antibiotics were included in the counting agar medium. [2] Tetracycline (8 μg/mL) was included in the counting agar medium. [3] Gentamicin (64 μg/mL) was included in the counting agar medium.

L. fermentum AGBG1 did not grow when cultured alone or co-cultured with *B. longum* BORI in the media containing tetracycline. The antimicrobial susceptibility test reported herein found that while *B. bifidum* BGN4 was very susceptible to tetracycline (MIC 1.0 μg/mL), *B. longum* BORI was resistant to tetracycline (MIC 64 μg/mL). However, the tetracycline resistance of *B. longum* BORI was not transferred to the recipient, *L. fermentum* AGBG1, in this study. *L. acidophilus* ATCC 4356, which is highly susceptible to gentamicin, grew well in normal MRS medium; however, *L. acidophilus* ATCC 4356 did not grow in the MRS medium containing gentamicin or the media that was co-cultured with *B. bifidum* BGN4 or *B. longum* BORI. By contrast, *B. bifidum* BGN4 and *B. longum* BORI showed resistance to 64 μg/mL gentamicin in this study. Therefore, this proves *B. bifidum* BGN4's resistance to gentamicin and *B. longum* BORI's resistance to gentamicin and tetracycline were not transferred to the recipient strains. It is worth noting that a 2011 report published by the Agency for Healthcare Research and Quality (AHRQ) [58] extensively reviewed 622 studies on six genera (i.e., *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Streptococcus*, *Enterococcus*, and *Bacillus* spp.), and found no clinical evidence of the theoretical possibility of gene transfer from probiotics to other microorganisms.

2.4.3. PCR Results on Antibiotic Resistance Genes

Even though the whole genome of *B. bifidum* BGN4 (Accession no.: CP001361.1) and *B. longum* BORI show that neither contain a plasmid capable of transferring the antibiotic-resistance gene, PCR analysis on ten antibiotic genes such as gentamicin(*aaac(6)–aph(2)*), kanamycin(*AphA3, aaaD*), streptomycin(*aadE*), trimethroprim(*dfrA*), and tetracycline(*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S)) were conducted. All the tested *Bifidobacterium* spp. in this study were identified using 16S rRNA *Bifidobacterium* genus specific primers (Figure 2). The PCR results on antibiotics genes are shown in Figure 3. There were no amplicons that indicate resistance genes in *B. bifidum* BGN4, *B. longum* BORI, and other *Bifidobacterium* spp. in this study.

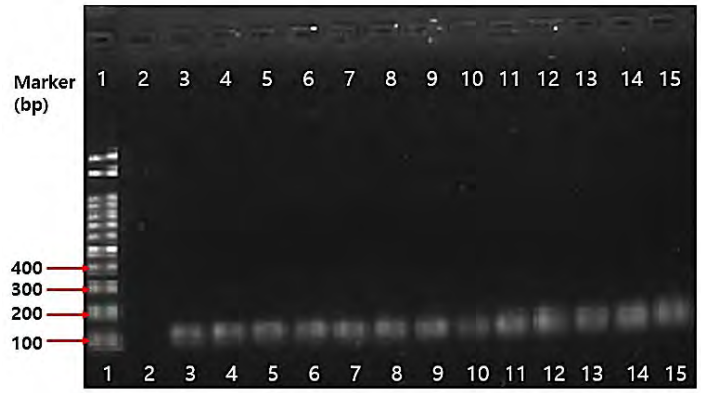


Figure 2. PCR analysis results of various *Bifidobacterium* spp.: Lane 1: marker; Lane 2: without loading; Lane 3: *B. lactis* AS60; Lane 4: *B. bifidum* KCTC 3440; Lane 5: *B. longum* BORI; Lane 6: *B. longum* KCCM 91563; Lane 7: *B. lactis* BB-12; Lane 8: *B. longum* RD47; Lane 9: *B. bifidum* BGN4; Lane 10: *B. thermophilum* KCCM 12097; Lane 11: *B. adolescentis* ATCC 15703; Lane 12: *B. lactis* AD011; Lane 13: *B. infantis* ATCC 15697; Lane 14: *B. breve* M-16V; Lane 15: *B. animalis* ATCC 25527.

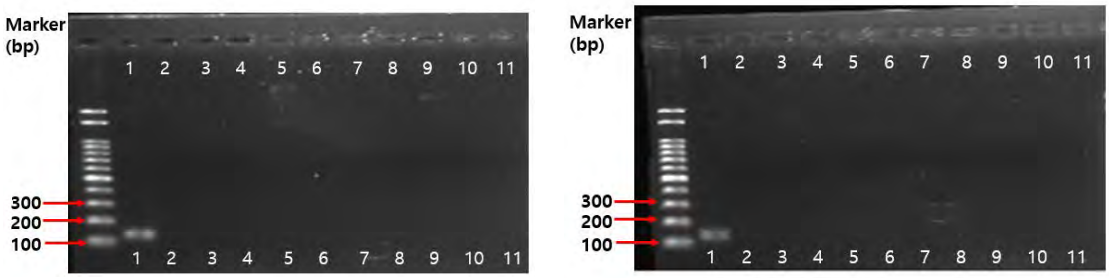


Figure 3. PCR analysis results of the antibiotic resistance gene in *B. bifidum* BGN4 and *B. longum* BORI: (**a**) *B. bifidum* BGN4; (**b**) *B. longum* BORI; Lane 1: *Bifidobacterium* genus-specific primers; Lane 2: gentamicin(*aaac(6)-aph(2)*), Lane 3: kanamycin(*AphA3*), Lane 4: streptomycin(*aadE*), Lane 5: trimethoprim(*dfrA*); Lane 6: tetracycline K(*tet*(K)); Lane 7: tetracycline L(*tet*(L)); Line 8: tetracycline M(*tet*(M)), Lane 9: tetracycline O(*tet*(O)), Lane 10: tetracycline S(*tet*(S)); Lane 11: kanamycin(*aaaD*).

Recently, the intrinsic gentamicin-resistance of *Bifidobacterium* spp. was putatively attributed to the presence of two genes, namely *Bbr_0651* and *Bbr_1586*, which are enzymes present in the *Bifidobacterium* chromosome DNA, with both coding for putative phosphotransferase enzymes [59]. Tetracycline resistance genes (*tet*) are widely distributed in the *Bifidobacterium* genus; however, it is known as a ribosomal protection protein [48,60]. The tetracycline W (*tet*(W)) gene was found in *B. longum* BORI chromosome DNA. In the study of Mättö et al. [54], human- and probiotic-associated *Bifidobacterium* species (203 strains) showed high MIC values for tetracycline (i.e., ≥16 mg/mL; prevalence of 4–18%) that were attributed to the presence of tetracycline genes (*tet*), where *tet*(W), and *tet*(O) were detected. The *tet*(W), and *tet*(M) were found in 26, and 7%, respectively, of the *Bifidobacterium* isolates. The role of the *tet*(W) gene is presumed to be the translation factor GTPase of the TRAFAC family, which induces a noncovalent modification to the ribosome that destroys the effect of tetracycline, inhibiting protein synthesis [61].

2.5. Mucin Degradation

The intestinal mucus gel layer is an important constituent of the intestinal barrier that consists of a glycoprotein family. Multiple groups have reported that bacterial translocation can occur in infants and immunocompromised hosts, even if the intestinal mucus acts as a biological shield from

microbes. This bacterial translocation has the potential to cause sepsis, and is one of the most serious probiotic safety concerns. Some scientists have also reported the possibility of bacteremia—endocarditis due to the administration of probiotic strains [62,63]. According to Ruas-Madiedo et al. [64], some *Bifidobacterium* spp. demonstrate mucolytic activities and have genes that induce mucin degrading enzymes. However, the majority of *Bifidobacterium* spp., such as *B. longum* and *B. pseudocatenulatum*, did not display mucolytic activity.

In order to confirm their microbial safety, it is necessary to evaluate translocation ability via mucolytic capacity analysis of each strain. In this study, the translocation capabilities of *B. bifidum* BGN4 and *B. longum* BORI were measured using in vitro mucolytic assays. The cell growth rates after incubation were examined in five kinds of modified MRS media by measuring their absorbances at 550 nm: basal medium (glucose-free MRS, ◇), basal medium with 0.5% mucin (×), 1.0% mucin (◯), 0.5% glucose (Δ), and 1.0% glucose (□) (Figure 4).

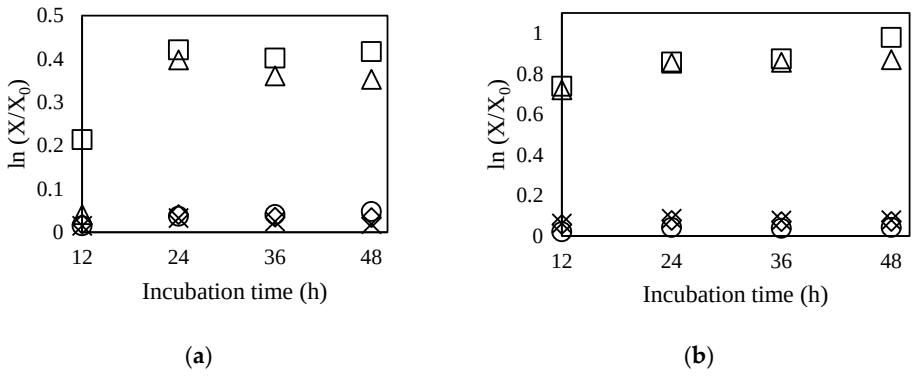


Figure 4. Growth curves of *B. bifidum* BGN4 (**a**) and *B. longum* BORI (**b**) in modified MRS with various carbon sources: basal medium (glucose-free MRS, ◇), basal medium with 0.5% mucin (×), 1.0% mucin (◯), 0.5% glucose (Δ), and 1.0% glucose (□).

In general, when simple sugars (e.g., glucose, fructose, maltose, and sucrose) are added, mucinase production can be inhibited due to catabolic repression. A false negative result can be obtained despite the microorganisms' potential to produce mucinolytic enzymes. Therefore, to obtain accurate data, glucose, which is generally used as a carbon source in the MRS medium, was intentionally removed from the medium in which the experimental cells were cultivated. If *B. bifidum* BGN4 and *B. longum* BORI were able to produce mucinase, they would be able to source carbon and grow actively through mucin digestion. As shown in Figure 2, the growth of both probiotic strains was actively induced when glucose was added as a carbon source. However, when mucin was added instead of glucose, no growth was observed in either strain. These observations clearly indicate that *B. bifidum* BGN4 and *B. longum* BORI did not use mucin as a carbon source for their growth. This study, as suggested by other studies [65,66], shows that neither *B. bifidum* BGN4 nor *B. longum* BORI degrade mucin, indicating that the strains are not capable of damaging intestinal surfaces and do not have translocational abilities.

2.6. Genetic Stability

The genetic variation of edible microorganisms possibly results in indels (i.e., gene deletion and insertion) and mutations. A critical consideration of commercializing probiotics is whether it is possible to maintain genetic safety over the long term. However, the genetic stability of commercial probiotic strains has not yet been reported. Theoretically, an evaluation of genetic stability requires the entire genome sequence of the strain.

The entire genome sequence of *B. bifidum* BGN4 has been published [31], and consists of a 2,223,664 bp circular chromosome (62.65% G+C) with no plasmids. A total of 1835 coding sequences (CDSs), 7 pseudogenes, 3 rRNA operons, and 52 tRNAs were compiled from the nucleotide

sequence. This study shows that the similarity in the genomic comparison between 1st generation and 25th generation samples were 99.9996~99.9998% via the Orthologous Average Nucleotide Identity (OrthoANI) value. (Table 5).

Table 5. OrthANI value

Strain/Sample	***B. bifidum*** **BGN4-1/13075.BBGN41.1** [1]	***B. bifidum*** **BGN4-2/13075.BBGN42.1** [2]	***B. bifidum*** **BGN4-3/13075.BBGN43.1** [3]
B. bifidum BGN4-1/13075.BBGN41.1 [1]	100	99.9997	99.9996
B. bifidum BGN4-2/13075.BBGN42.1 [2]	99.9997	100	99.9998
B. bifidum BGN4-3/13075.BBGN43.1 [3]	99.9996	99.9998	100

[1] *B. bifidum* BGN4-1/13075.BBGN41.1 denotes the 1st generation; [2] *B. bifidum* BGN4-2/13075.BBGN42.1 denotes the 25th generation; [3] *B. bifidum* BGN4-3/13075.BBGN43 and *B. bifidum* BGN4-2 are the 25th generations.

The difference between 0.0002% and 0.0004% is equivalent to 4.4 to 8.8 bp mutation of the entire nucleotide sequence, which can be assumed to be due to sequencing errors or spontaneous evolutionary mutations. Therefore, it is concluded that there was little genetic mutation, and the genetic information did not change in the process of cultivating 25 generations.

2.7. Virulence Factors

The genome sequences of *B. bifidum* BGN4 and *B. longum* BORI were compared with the genome sequences of four well-known pathogens (*E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus*). The virulence factors included *E. coli* Shiga toxin gene and *S. aureus* exoenzyme genes, host immune alteration or evasion genes and toxin genes. No virulence factors were found in the genomic sequences of *B. bifidum* BGN4 and *B. longum* BORI. Thus, this result shows that the genomic sequences of *B. bifidum* BGN4 and *B. longum* BORI do not include toxic or pathogenic genes related to *E. coli*, *Enterococcus*, *Listeria*, and *S. aureus*.

3. Materials and Methods

3.1. Microorganisms

The bacterial strains, including origin, culture medium, and test methods used in this study are presented in Table 6.

Table 6. Strain list and methods.

Strains	Origin	Medium	Method
Bifidobacterium bifidum BGN4	BIFIDO Co., Ltd. (Hongcheon, Korea)	BHI [1], Blood agar [2], whole milk [3], LSM-Cys [4], MRS [5–8]	3.2., 3.3., 3.4., 3.5.2., 3.5.3., 3.5.4., 3.6., 3.7
Bifidobacterium longum BORI	BIFIDO Co., Ltd. (Hongcheon, Korea)	BHI [1], Blood agar [2], MRS [3,5–7], LSM-Cys [4]	3.2., 3.3., 3.4., 3.5.2., 3.5.4., 3.6
Bacteroides fragilis ATCC 25285	American Type Culture Collection (Manassas, VA, USA)	BHI [1]	3.2
Bacteroides thetaiotaomicron ATCC 29741	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Bifidobacterium adolescentis ATCC 15703	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium animalis ATCC 25527	American Type Culture Collection (Manassas,VA, USA)	MRS [6]	3.5.4
Bifidobacterium animalis subsp. *lactis* AD011	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4

Table 6. *Cont.*

Strains	Origin	Medium	Method
Bifidobacterium animalis subsp. *lactis* AS60	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4
Bifidobacterium animalis subsp. *lactis* BB-12	Isolated from a pharmaceutical product, USA	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium bifidum KCTC 3440	Korean Collection for Type Cultures, (Jeongeup, Korea)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium breve ATCC 15701	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Bifidobacterium breve M-16V	Isolated from a pharmaceutical product, USA	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium infantis ATCC 15697	American Type Culture Collection (Manassas, VA, USA)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium longum ATCC 15707	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4]	3.5.2
Bifidobacterium longum BB536	Isolated from a pharmaceutical product, USA	LSM-Cys [4]	3.5.2
Bifidobacterium longum KCCM 91563	Korean Culture Center of Microorganisms (Seoul, Korea)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium longum RD47	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4
Bifidobacterium thermophilum KCCM 12097	Korean Culture Center of Microorganisms (Seoul, Korea)	MRS [6]	3.5.4
Clostridium perfringens ATCC 13124	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Enterococcus faecalis ATCC 29212	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4]	3.5.2
Enterobacter cloacae subsp. *cloaca* ATCC 13047	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Enterobacter faecalis ATCC 19433	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Lactobacillus acidophilus ATCC 4356	American Type Culture Collection (Manassas,VA, USA)	MRS [5]	3.5.3
Lactobacillus fermentum AGBG1	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [5]	3.5.3
Lactobacillus plantarum KFRI 708	Korea Food Research Institute (Wanju, Korea)	BHI [1]	3.2
Listeria ivanovii subsp. *ivanovii* ATCC 19119	American Type Culture Collection (Manassas,VA, USA)	Blood Agar [2]	3.3

[1] Ammonia production test (3.2.): *B. bifidum* BGN4, *B. longum* BORI, *B. breve* ATCC 15701, *L. plantarum* KFRI 708, *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. perfringens* ATCC 13124, *E. cloacae* ATCC 13047, and *E. faecalis* ATCC 19433 were anaerobically cultured in brain heart infusion (BHI) (BD BBL™, Franklin Lakes, NJ, USA) medium at 37 °C for 5 days. [2] Hemolytic test (3.3): *B. bifidum* BGN4 and *B. longum* BORI were anaerobically cultured in Blood agar (BHI broth medium supplemented with 1.5% agar and 5% sheep blood) at 37 °C for 2 days. *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, a positive control for hemolysis, was aerobically cultivated in Blood agar at 37 °C for 2 days. [3] Biogenic amine production test (3.4): *B. bifidum* BGN4 and *B. longum* BORI, were anaerobically cultured in whole milk (Seoul Milk, Seoul, Korea) or de Man–Rogosa–Sharpe (MRS) broth (BD Difco™, Franklin Lakes, NJ, USA) with supplementation of 0.05% (*w*/*w*) L-cysteine-HCl (Sigma, St. Louis, MO, USA) at 37 °C for 15 h. [4] Antimicrobial susceptibility test (3.5.2.): LSM-Cys broth medium supplemented with 0.03% L-cysteine-HCl, which is composed with 90% of IST and 10% of MRS broth medium. [5] Antibiotic resistance transferability test (3.5.3.): *Bifidobacterium* strains were anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl and *Lactobacillus* strains were cultured without L-cysteine-HCl at 37 °C for 18 h. [6] PCR assay on antibiotic resistance gene (3.5.4.): *Bifidobacterium* strains were anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl at 37 °C for 18 h. [7] Mucin degradation test (3.6.): *B. bifidum* BGN4 and *B. longum* BORI were anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl at 37 °C for 48 h. [8] Genetic stability test (3.7.): *B. bifidum* BGN4 was anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl.

3.2. Ammonia Production Test

B. bifidum BGN4, *B. longum* BORI, *B. breve* ATCC 15701, *L. plantarum* KFRI 708, *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. perfringens* ATCC 13124, *E. cloacae* ATCC 13047, and *E. faecalis*

ATCC 19433 were anaerobically cultured in brain heart infusion (BHI) (BD BBL™, Franklin Lakes, NJ, USA) media at 37 °C for 5 days. The production of ammonia by catalyzed indophenol reaction was determined according to the method of Chaney and Marbach [67]. To evaluate the generated extracellular ammonia levels, the media supernatants of each strain were obtained by centrifuging at 10,000× *g* at 4 °C for 30 min. The media was then adjusted to pH 7 using 1 N NaOH. Two solutions were prepared as follows: Solution 1 consisted of 2 g phenol and 0.01 g sodium nitroferricyanide dehydrate dissolved in 200 mL distilled water and Solution 2 consisted of 1 g sodium hydroxide and 0.08 g sodium hypochlorite dissolved in 200 mL distilled water. Aliquots (10 µL) of Solutions 1 and 2 were added to 96 well plates with 100 µL of the media supernatants of each strain. Three replications of this test were conducted on each strain. The 96 well plates were maintained at room temperature for one hour, and the absorbance was measured at 625 nm. Bacteria-free BHI medium was used as a negative control and the ammonia concentration was calculated using a standard curve.

3.3. Hemolytic Test

B. bifidum BGN4 and *B. longum* BORI were anaerobically cultured in blood agar (BHI broth medium supplemented with 1.5% agar and 5% sheep blood) at 37 °C for 2 days. *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, a positive control for hemolysis, was aerobically cultivated in blood agar at 37 °C for 2 days. The plates were then analyzed for the presence or absence of microbial hemolysis properties by holding the plate up to a light source and viewing through both sides of the plate. Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any hemolysis on the blood plates (γ-hemolysis) were considered non-hemolytic. Strains that displayed blood lyses zones (white-hued zones) around the colonies were classified as microorganisms with hemolytic (β-hemolysis) properties.

3.4. Biogenic Amine Production Test

B. bifidum BGN4 and *B. longum* BORI were anaerobically cultured in whole milk (Seoul Milk, Korea) or de Man–Rogosa–Sharpe (MRS) broth (BD Difco™, Franklin Lakes, NJ, USA)) with supplementation of 0.05% (*w*/*w*) L-cysteine-HCl (Sigma, St. Louis, MO, USA) at 37 °C for 15 h. Four biogenic amines (cadaverine (≥97.0%, Cat. #33211), histamine (≥97.0%, Cat. #H7125), putrescine (≥98.5%, Cat. #51799), and tyramine (99%, Cat. #T90344)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,7-Diaminoheptane (internal standard; ISTD, 98%, Cat. #D174708), dansyl chloride (≥99.0%, Cat. #39220), and L-proline (≥99.0%, Cat. #P0380) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Whatman No. 4 filter paper was obtained from Whatman Intl., Ltd. (Maidstone, UK). Sodium carbonate (99.0%, Cat. #433401201), ether (99.0%, Cat. #33475S1280), and acetone (99.7%, Cat. #A0108) were obtained from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea).

The biogenic amine analysis extraction procedure was conducted as described by Kim and Ji [68]. Each 5 g sample was weighed and vortexed with 25 mL of 0.1 N HCl for 5 min. After the resulting homogenate was centrifuged at 10,000× *g* for 15 min at 4 °C (2236R high-speed centrifuge; Labogene Aps, Lillerød, Denmark), the aqueous layer was collected, and the residue was re-extracted as described above. The collected extracts were filtered through Whatman No. 4 filter paper. One milliliter of each extract was transferred to a glass test tube, and the following was added: 0.1 mL of internal standard (1,7-diaminoheptane, 100 mg/L), 0.5 mL of saturated sodium carbonate, and 1 mL of 1% dansyl chloride in acetone. After thoroughly mixing, the test tubes were incubated in a dark water bath (WBC 1510A; Jeio Tech. Co., Ltd., Seoul, Korea) at 45 °C for 60 min. Subsequently, 0.5 mL of 10% proline and 5 mL ether were added to each sample and allowed to rest for 5 min to remove the residual dansyl chloride. The supernatants were suspended and evaporated (Scanvac Speed Vacuum Concentrator; Labogene Aps, Lillerød, Denmark) at 20 °C until dry. The dry residue was diluted with 1 mL of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA). The reconstituted sample and standard were filtered through a 0.2 µm syringe filter for HPLC analysis. The HPLC analysis of the biogenic amines was performed at the National Instrumentation Center for Environmental Management (NICEM) at

Seoul National University (Seoul, Korea). The HPLC determinations were performed as described in Table 7.

Table 7. HPLC conditions.

Parameters	Conditions		
HPLC	Thermo Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, St Peters, MO, USA)		
Column	VDSpher C-18 column (4.6 × 250 mm, 5 µm) (VDS optilab Chromatographietechnik GmbH, Berlin, Germany)		
Mobile solvent	Time (min)	Distilled Water (%)	Acetonitrile (%)
	0	40	60
	1	40	60
	20	0	100
	25	0	100
	26	40	60
	30	40	60
Flow rate	0.8 mL		
Column temperature	30 °C		
Injection volume	20 µL		
Detector	UV 250 nm		

3.5. Antimicrobial Susceptibility and Antibiotic Resistance Transferability Test

3.5.1. Antimicrobial Agents

Twenty antimicrobial agents were used: ampicillin sodium salt (Sigma, Lot#BCBW1243), carbenicillin disodium salt (Sigma, Lot#116M4834V), cephalothin sodium salt (Sigma Lot#056M4858V), chloramphenicol (Sigma, Lot#SLBR8869V), clindamycin hydrochloride (Sigma, Lot#021M1533), dicloxacillin sodium salt hydrate (Sigma, Lot#SZBD263XV), erythromycin (Sigma, Lot#WXBC4044V), gentamicin sulfate (Sigma, Lot#SLBP3082V), kanamycin sulfate (Sigma, Lot#066M4019V), metronidazole (Sigma, Lot#MKBZ3056V), mupirocin (Sigma, Lot#106M4733V), neomycin sulfate (Sigma, Lot#LRAB3300), penicillin G (Sigma, Lot#087M4834V), phosphomycin disodium salt (Sigma, Lot#096M4031V), polymyxin B sulfate salt (Sigma, Lot#027M4002V), rifampicin (Sigma, Lot#MKCC2435), streptomycin sulfate salt (Sigma, Lot#SLBT8451), tetracycline (Sigma, Lot#126M4769V), trimethoprim–sulfamethoxazole (trimethoprim (Sigma, Lot#097M4017V), sulfamethoxazole (Sigma, Lot#BCBT3855)), vancomycin hydrochloride (USP, Lot#R07250). vancomycin hydrochloride was purchased from USP (Rockville, MD, USA), and the remaining 19 antimicrobiotics were purchased from Sigma (St. Louis, MO, USA). Each of the antibiotic powders was dissolved and diluted in appropriate diluents and filter sterilized prior to addition to LSM-Cys broth medium, composed of 90% of IST and 10% of MRS broth medium. IST broth was purchased from KisanBio Co., Ltd. (Mbcell Iso-Sensitest Broth, Seoul, Korea) and MRS was purchased from Becton, Dickinson and Company (BD Difco™ MRS Lactobacilli broth, Franklin Lakes, NJ, USA). Serial dilutions of antimicrobial agents ranging from 1024 to 0.0032 µg/mL were prepared.

3.5.2. Antimicrobial Susceptibility Test

Minimal inhibitory concentration (MIC) values for all bacterial isolates were determined by the ISO 10932:2010 broth microdilution procedure [69]. The LSM-Cys broth medium supplemented with 0.03% (*w*/*v*) L-cysteine HCl containing antibiotics at different concentrations was used to prepare each well of a microwell plate. The inoculum was adjusted to a turbidity equivalent to 0.16 to 0.2 at 625 nm as measured by a Hitachi Spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). The solution corresponded to approximately 3×10^8 cfu/mL. Each inoculum was added to a double strength LSM-Cys broth medium at a rate of 0.2%. A 50 µL diluted bacterial suspension was added to each well; no negative control well was employed. The microdilution plates were prepared with a series of twofold dilutions of antibiotics. The microdilution plates were incubated at 37 °C for 48 h in an anaerobic (5% CO_2, 10% H_2 and 85% N_2) chamber. The MIC was defined as the lowest concentration

of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well. The experiments were replicated three times.

3.5.3. Antibiotic Resistance Transferability Test

Conjugal transfer of antibiotic resistance was assessed via the methods of Tannock [70]. Equal bacterial cell volumes (1 mL) of the donor and recipient strains were mixed and centrifuged at 7000× *g* for 10 min (2236R high-speed centrifuge; Labogene Aps, Lillerød, Denmark) (see Table 8). After disposing of the supernatant, the bacterial cell pellet was resuspended in the MRS broth medium and cultivated at 37 °C for 12 h in an anaerobic chamber. The collected bacterial cells were filtered through a 0.45 μm microfilter membrane (Whatman Intl., Ltd., Maidstone, UK) and the membrane was placed on the surface of MRS agar and incubated anaerobically at 37 °C for 24 h. The bacterial cells were washed with 4 mL of 0.9% sterile saline, diluted to 10^{-3}, 10^{-4}, and 10^{-5}, respectively, and then plated on MRS agar containing gentamicin or tetracycline. The plates were incubated aerobically or anaerobically at 37 °C for 36 h. Three replicates of all experiments were conducted.

Table 8. Test scheme.

Donor Strains Recipient Strains	*B. bifidum* BGN4	*B. longum* BORI
L. fermentum AGBG1	N/A [1]	BORI + AGBG1
L. acidophilus ATCC 4356	BGN4 + ATCC 4356	BORI + ATCC 4356

[1] N/A denotes not applicable because *B. bifidum* BGN4 was highly susceptible to tetracycline, which resulted in no growth on the media containing tetracycline.

3.5.4. PCR Assay on Antibiotic Resistance Genes

The experimental conditions of Guo et al. [71] were used for these tests. The genomic DNA of the pure culture bacteria was extracted using MG™ Cell Genomic DNA Extraction SV miniprep (MGmed, Seoul, Korea). The extraction was performed according to the manufacturers' instructions, and the total bacterial DNA was eluted with 200 μL of sterile water. To ensure that the ratio of absorbance at 260 nm to absorbance at 280 nm was 1.8–2.0., DNA extracts were aliquoted and stored at −20 °C. Polymerase chain reactions (PCR) were used to detect antibiotic resistance genes by gene-specific primers (Table 9). The following reaction mixture was added to each sample: 1.5 μL DNA (50 ng), 2 μL primer (100 pmol), dNTP mixture 8 μL, 2XGC buffer I, and adjusted to 50 μL volume by sterilized distilled water. The amplification program was an initial denaturation step of 94 °C for 5 min, and then 30 cycles of: 94 °C for 30 s, annealing temperature (Table 9) for 30 s, 72 °C for 1 min, and 72 °C for 7 min. The amplicons were analyzed on 1.5% agarose gel to confirm the DNA fragment size.

Table 9. Primers and conditions for PCR detection [1].

No.	Primer Name		Oligo Sequence	TM (°C)	Product Size	Reference
1	*Bifidobacterium* genus-specific primers	-	F: 5′-TCGCGTCYGGTGTGAAAG-3′ R: 5′-GGTGTTCTTCCCGATATCTACA-3′	55	128 bp	[72]
2	Gentamicin	*aaac(6)-aph(2)*	F: 5′-CCAAGAGCAATAAGGGCATA-3′ R: 5′-CACTATCATAACCACTACCG-3′	60	220 bp	[73]
3	Kanamycin	*AphA3*	F: 5′-GCCGATGTGGATTGCGAAAA-3′ R: 5′-GCTTGATCCCCAGTAAGTCA-3′	52	292 bp	[74]
4	Streptomycin	*aadE*	F: 5′-ATGGAATTATTCCCACCTGA-3′ R: 5′-TCAAAACCCCTATTAAAGCC-3′	50	565 bp	[74]
5	Trimethoprim	*dfrA*	F: 5′-AAAAGGGGCAGAGCATG-3′ R: 5′-AGAAAATGGCGTAATCGGTA-3′	50	474 bp	[75]
6	Tetracycline(K)	*tet*(K)	F: 5′-TTAGGTGAAGGGTTAGGTCC-3′ R: 5′-GCAAACTCATTCCAGAAGCA-3′	55	169 bp	[76]
7	Tetracycline(L)	*tet*(L)	F: 5′-GTTGCGCGCTATATTCCAAA-3′ R: 5′-TTAAGCAAACTCATTCCAGC-3′	55		
8	Tetracycline(M)	*tet*(M)	F: 5′-GTTAAATAGTGTTCTTGGAG-3′ R: 5′-CTAAGATATGGCTCTAACAA-3′	55	401 bp	[77]
9	Tetracycline(O)	*tet*(O)	F: 5′-GATGGCATACAGGCACAGAC-3′ R: 5′-CAATATCACCAGAGCAGGCT-3′	55		
10	Tetracycline(S)	*tet*(S)	F: 5′-TGGAACGCCAGAGAGGTATT-3′ R: 5′-ACATAGACAAGCCGTTGACC-3′	55	1923 bp	[78]
11	Kanamycin	*aaaD*	F: 5′-TGCGTTTTGACACATCCAC-3′ R: 5′-GGTGTTTATGGCTCTCTTGG-3′	55		

[1] The experiment conditions are secondary quoted from Guo et al. [71].

3.6. Mucin Degradation Test

Partially purified Mucin from porcine stomach—Type III, was purchased from Sigma (St. Louis, MO, USA). An MRS broth medium without a carbon source (i.e., basal medium containing yeast extract 0.75% (*w*/*v*), soy peptone 0.25% (*w*/*v*), fish extract 0.25% (*w*/*v*), sodium acetate 0.25% (*w*/*v*), ammonium citrate 0.1% (*w*/*v*), sodium phosphate monobasic 0.05% (*w*/*v*), sodium phosphate dibasic 0.025% (*w*/*v*), Tween 80 0.05% (*w*/*v*), L-cysteine HCl 0.05% (*w*/*v*), maleic acid 0.005% (*w*/*v*), taurine 0.00625% (*w*/*v*), magnesium sulfate 0.005% (*w*/*v*), manganese sulfate 0.0025% (*w*/*v*), and distilled water 98.2% (*v*/*v*)) was used as a negative control. To each of the four MRS broth media, 0.5% (*w*/*v*) mucin, 1.0% (*w*/*v*) mucin, 0.5% (*w*/*v*) glucose, and 1% (*w*/*v*) glucose were added. After the inoculation of the microorganisms in each MRS medium, the samples were cultured at 37 °C for 48 h under anaerobic conditions. After incubation, the bacterial growth was assessed by measuring absorbance at 550 nm at 12, 24, 36, and 48 h. The initial optical density value of the media was subtracted from the final value for each test sample.

3.7. Genetic Stability Test

B. bifidum BGN4 was plated on a MRS agar plate via streaking from a stock stored at −80 °C and incubated anaerobically at 37 °C for 24 h to obtain a single colony. A single colony was inoculated into 10 mL of MRS broth supplemented with 0.05% (*w*/*v*) L-cysteine HCl and regarded as the 2^0 (1st) generation (about 10^6 CFU/mL) of *B. bifidum* BGN4. *B. bifidum* BGN4 was incubated at 37 °C for about 12 h under anaerobic conditions to reach about 10^9 cfu/mL and obtain 210 generations. In the second subculture, 0.1 mL (1% inoculation, about 10^6 cfu/mL) of the primary culture was inoculated with 10 mL of MRS broth and cultured under the same conditions to obtain 220 generations of *B. bifidum* BGN4. For the third subculture, 0.1 mL (1% inoculation, approximately 10^6 CFU/mL) of the secondary culture was inoculated with 10 mL of MRS broth and incubated to 10^7 or 10^8 CFU/mL to obtain 2^{25} generations of *B. bifidum* BGN4. The viable count during cultivation was measured to confirm the generation number. The genomic DNA of the pure culture bacteria was extracted using MG™ Cell Genomic DNA Extraction SV Miniprep (MGmed, Seoul, Korea), according to the manufacturer's instructions. Whole genome sequencing and analysis were completed using an Illumina MiSeq sequencer and a Nextera XT Library Preparation kit (Illumina, San Diego, CA, USA). Nextera XT sequencing indices were used for multiplexing, and the participants were free to choose any sample index combination. The run acceptance criteria were a sequencing output of 5.6 Gb (to achieve an average sequencing coverage of 100-fold for the 20 samples with genome sizes of 2.8 Mb) and a Q30 read quality score of 75% [79]. The bioinformatics analysis was performed using Miseq raw data, and the comparative genomics analysis was completed with three Miseq raw data sets in ChunLab Co., Ltd. (Seoul, Korea).

3.8. Virulence Factors Researching

The search for virulence factors in *B. bifidum* BGN4 and *B. longum* BORI was completed using the VirulenceFinder1.5 Server, which is a component of the publicly available web-based tool for whole-genome sequencing(WGS) analysis hosted by the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/). The database system is designed to detect homologous sequences for the virulence genes related to *E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* in WGS data [80]. The output consists of best-matching genes from BLAST analysis of the selected database against the submitted genome of *B. bifidum* BGN4 or *B. longum* BORI. The selected %ID threshold was set at 90.00%, and the selected minimum length was set at 60%. If there is a matching result, the output shows information on the predicted virulence gene, the % ID, the length of query and database gene, the position of the hit in the contig, and the accession number of the hit.

4. Conclusions

Although probiotics have been widely used for their health benefits in food markets around the world, safety issues, including the side effects of probiotics, should be considered even more carefully than their clinical effects on consumers' health. In this study, it is shown that *B. bifidum* BGN4 and *B. longum* BORI did not produce ammonia or biogenic amines such as histamine, tyramine, or cadaverine. A trace amount of putrescine was found in both strains; however, the quantities were similar to or less than the amount detected in various foods regularly consumed. Neither probiotic demonstrated hemolysis activity nor mucin degrading activity. Their resistance to antibiotics, however, was not transferable in this study. These finding suggest that *B. bifidum* BGN4 and *B. longum* BORI are suitable for use in foods with little risk of harmful effects on the consumer.

Author Contributions: M.J.K. and S.K. designed the experiment under the supervision of M.S.P., G.E.J. and S.Y.K., H.H.L., H.J., S.K. and R.L. performed the microbiology experiments under the mentorship of M.S.P. and G.E.J. M.J.K., S.K., M.S.P. and G.E.J. performed the literature research together. M.J.K., S.K., M.S.P. and G.E.J. designed the research template. S.K. and T.V.J. edited and revised the manuscript based on a non-disclosure research agreement between Middle Tennessee State University and BIFIDO Co., Ltd. All authors discussed drafts and approved the final manuscript for publication.

Acknowledgments: This work was supported by Health and Welfare (03-PJI-PG11-VN01-SV04-0018), Republic of Korea, and the Promoting Regional specialized Industry (R0004140), the Ministry of Trade, Industry and Energy (MOTIE) and Korea Institute for Advancement of Technology (KIAT), the National Research Foundation of Korea (NRF) grant (No. 2017R1A2B2012390) funded by the Korea government (MSIP), the Bio &Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2017M3A9F3041747) and the Faculty Research and Creative Activity Committee (FRCAC) grant (No. 221745) funded by Middle Tennessee State University (MTSU). The authors wish to thank Lauren B. Mallet at Purdue University, Fred Marino and Karen M. Lauer at MTSU for their review and feedback.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lilly, D.M.; Stillwell, R.H. Probiotics: Growth-Promoting Factors Produced by Microorganisms. *Science* **1965**, *147*, 747–748. [CrossRef] [PubMed]
2. Probiotics: In Depth. Available online: https://nccih.nih.gov/health/probiotics/introduction.htm (accessed on 3 April 2018).
3. Food and Agriculture Organization-World Health Organization (FAO/WHO). Report on Joint FAO/WHO Guidelines for the Evaluation of Probiotics in Food. 2002. Available online: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf (accessed on 3 April 2018).
4. European Food and Feed Cultures Association (EFFCA). *Definition of Microbial Food Culture (MFC)*; EFFCA: Brussels, Belgium, 2003. Available online: http://www.effca.org/content/food-culture (accessed on 3 April 2018).
5. Akhter, N.; Wu, B.; Memon, A.M.; Mohsin, M. Probiotics and Prebiotics Associated with Aquaculture: A Review. *Fish Shellfish Immunol.* **2015**, *45*, 733–741. [CrossRef] [PubMed]
6. Ford, A.C.; Quigley, E.M.; Lacy, B.E.; Lembo, A.J.; Saito, Y.A.; Schiller, L.R.; Soffer, E.E.; Spiegel, B.M.; Moayyedi, P. Efficacy of Prebiotics, Probiotics and Synbiotics in Irritable Bowel Syndrome and Chronic Idiopathic Constipation: Systematic Review and Meta-Analysis. *Am. J. Gastroenterol.* **2014**, *109*, 1547–1561. [CrossRef] [PubMed]
7. O'Hara, A.M.; Shanahan, F. The Gut Flora as a Forgotten Organ. *EMBO Rep.* **2006**, *7*, 688–693. [CrossRef] [PubMed]
8. Sánchez, B. Bile Acid–Microbiota Crosstalk in Gastrointestinal Inflammation and Carcinogenesis: A Role for Bifidobacteria and Lactobacilli? *Nat. Rev. Gastroenterol Hepatol.* **2018**, *15*, 205. [CrossRef] [PubMed]
9. Lukjancenko, O.; Ussery, D.W.; Wassenaar, T.M. Comparative genomics of *Bifidobacterium*, *Lactobacillus* and Related Probiotic Genera. *Microb. Ecol.* **2012**, *63*, 651–673. [CrossRef] [PubMed]
10. Global Market Insights Inc. Probiotics Market Size to Exceed USD 64 Billion by 2023: Global Market Insights Inc. Available online: https://www.prnewswire.com/news-releases/probiotics-market-size-to-exceed-usd-64-billion-by-2023-global-market-insights-inc-578769201.html (accessed on 3 April 2018).

11. EFSA. Update of the List of QPS-Recommended Biological Agents Intentionally Added to Food or Feed as Notified to EFSA 4: Suitability of Taxonomic Units Notified to EFSA until March 2016. Available online: https://www.efsa.europa.eu/en/efsajournal/pub/4522 (accessed on 3 April 2018).
12. Anadón, A.; Martínez-Larrañaga, M.R.; Ares, I.; Martínez, M.A. *Nutraceuticals; Efficacy, Safety and Toxicity. Chapter 55—Probiotics: Safety and Toxicity Considerations*; Hill-Parks, E., Ed.; Academic Press: London, UK, 2016; pp. 777–798, ISBN 978-0-12-802147-7.
13. Huys, G.; Botteldoorn, N.; Delvigne, F.; de Vuyst, L.; Heyndrickx, M.; Pot, B.; Dubois, J.J.; Daube, G. Microbial Characterization of Probiotics-Advisory Report of the Working Group "8651 Probiotics" of the Belgian Superior Health Council (SHC). *Mol. Nutr. Food Res.* **2013**, *57*, 1479–1504. [CrossRef] [PubMed]
14. Shokryazdan, P.; Jahromi, M.F.; Liang, J.B.; Kalavathy, R.; Sieo, C.C.; Ho, Y.W. Safety Assessment of Two New Lactobacillus Strains as Probiotic for Human Using a Rat Model. *PLoS ONE* **2016**, *11*, e0159851. [CrossRef] [PubMed]
15. Tan, Q.; Xu, H.; Aguilar, Z.P.; Peng, S.; Dong, S.; Wang, B.; Li, P.; Chen, T.; Xu, F.; Wei, H. Safety Assessment and Probiotic Evaluation of *Enterococcus faecium* Isolated from Sourdough. *J. Food Sci.* **2013**, *78*, M587–M593. [CrossRef] [PubMed]
16. Endres, J.R.; Clewell, A.; Jade, K.A.; Farber, T.; Hauswirth, J.; Schauss, A.G. Safety Assessment of a Proprietary Preparation of a Novel Probiotic, *Bacillus coagulans*, as a Food Ingredient. *Food Chem. Toxicol.* **2009**, *47*, 1231–1238. [CrossRef] [PubMed]
17. Sanders, M.E.; Akkermans, L.M.A.; Haller, D.; Hammerman, C.; Heimbach, J.; Hörmannsperger, G.; Huys, G.; Levy, D.D.; Lutgendorff, F.; Mack, D.; et al. Safety Assessment of Probiotics for Human Use. *Gut Microbes.* **2010**, *1*, 164–185. [CrossRef] [PubMed]
18. Salyers, A.A.; Gupta, A.; Wang, Y. Human Intestinal Bacteria as Reservoirs for Antibiotic Resistance Genes. *Trends Microbiol.* **2004**, *12*, 412–416. [CrossRef] [PubMed]
19. Sommer, M.O.A.; Dantas, G.; Church, G.M. Functional Characterization of the Antibiotic Resistance Reservoir in the Human. *Science* **2009**, *325*, 1128–1131. [CrossRef] [PubMed]
20. Fouhy, F.; Guinane, C.M.; Hussey, S.; Wall, R.; Ryan, C.A.; Dempsey, E.M.; Murphy, B.; Ross, R.P.; Fitzgerald, G.F.; Stanton, C.; et al. High-Throughput Sequencing Reveals the Incomplete, Short-Term, Recovery of the Infant Gut Microbiota Following Parenteral Antibiotic Treatment with Ampicillin and Gentamicin. *Antimicrob. Agents. Chemother.* **2012**, *56*, 5811–5820. [CrossRef] [PubMed]
21. Fallani, M.; Young, D.; Scott, J.; Norin, E.; Amarri, S.; Adam, R.; Aguilera, M.; Khanna, S.; Gil, A.; Edwards, C.A.; et al. Intestinal microbiota of 6-week-old infants across Europe: Geographic influence beyond delivery mode, breast-feeding, and antibiotics. *J. Pediatr. Gastroenterol. Nutr.* **2010**, *51*, 77–84. [CrossRef] [PubMed]
22. Murphy, E.F.; Cotter, P.D.; Healy, S.; Marques, T.M.; O'Sullivan, O.; Fouhy, F.; Clarke, S.F.; O'Toole, P.W.; Quigley, E.M.; Stanton, C.; et al. Composition and energy harvesting capacity of the gut microbiota: Relationship to diet, obesity and time in mouse models. *Gut* **2010**, *59*, 1635–1642. [CrossRef] [PubMed]
23. Imperial, I.C.V.J.; Ibana, J.A. Addressing the Antibiotic Resistance Problem with Probiotics: Reducing the Risk of Its Double-Edged Sword Effect. *Front. Microbiol.* **2016**, *7*, 1983. [CrossRef] [PubMed]
24. O'Callaghan, A.; van Sinderen, D. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Front. Microbiol.* **2016**, *7*, 925. [CrossRef] [PubMed]
25. Park, M.S.; Kwon, B.; Ku, S.; Ji, G.E. The Efficacy of *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031 Probiotic Treatment in Infants with Rotavirus Infection. *Nutrients* **2017**, *9*, 887. [CrossRef] [PubMed]
26. Seo, J.M.; Ji, G.E.; Cho, S.H.; Park, M.S.; Lee, H.J. Characterization of a *Bifidobacterium longum* BORI Dipeptidase Belonging to the U34 Family. *Appl. Environ. Microbiol.* **2007**, *73*, 5598–5606. [CrossRef] [PubMed]
27. JI, G.E. Development of *Bifidobacterium* sp. BGN4 and BORI with Novel Probiotic Activity. *Int. Meet. Microbiol. Soc. Korea* **2005**, *5*, 81–84.
28. Ku, S.; Park, M.S.; Ji, G.E.; You, H.J. Review on *Bifidobacterium bifidum* BGN4: Functionality and Nutraceutical Applications as a Probiotic Microorganism. *Int. J. Mol. Sci.* **2016**, *17*, 1544. [CrossRef] [PubMed]
29. Ku, S.; You, H.J.; Ji, G.E. Enhancement of Anti-Tumorigenic Polysaccharide Production, Adhesion, and Branch Formation of *Bifidobacterium bifidum* BGN4 by Phytic Acid. *Food Sci. Biotechnol.* **2009**, *18*, 749–754.

30. Health Functional Food Code (HFFC). II.2.51. Probiotics. Ministry of Food and Drug Safety in Korea. 2010. Available online: http://www.mfds.go.kr/files/upload/eng/4.Health_Functioanl_Food_Code_(2010.09).pdf (accessed on 5 April 2018).
31. Yu, D.S.; Jeong, H.; Lee, D.H.; Kwon, S.K.; Song, J.Y.; Kim, B.K.; Park, M.S.; Ji, G.E.; Oh, T.K.; Kim, J.F. Complete Genome Sequence of the Probiotic Bacterium *Bifidobacterium bifidum* strain BGN4. *J. Bacteriol.* **2012**, *194*, 4757–4758. [CrossRef] [PubMed]
32. Igai, K.; Itakura, M.; Nishijima, S.; Tsurumaru, H.; Suda, W.; Tsutaya, T.; Tomitsuka, E.; Tadokoro, K.; Baba, J.; Odani, S.; et al. Nitrogen Fixation and *nif*H Diversity in Human Gut Microbiota. *Sci. Rep.* **2016**, *6*. [CrossRef] [PubMed]
33. Smith, E.A.; Macfarlane, G.T. Formation of Phenolic and Indolic Compounds by Anaerobic Bacteria in the Human Large Intestine. *Microbiol. Ecol.* **1997**, *33*, 180–188. [CrossRef]
34. Vince, A.J.; Burridge, S.M. Ammonia Production by Intestinal Bacteria: The Effects of Lactose, Lactulose and Glucose. *J. Med. Microbiol.* **1980**, *13*, 177–191. [CrossRef] [PubMed]
35. Zarei, M.; Najafzadeh, H.; Enayati, A.; Pashmforoush, M. Biogenic Amines Content of Canned Tuna Fish Marketed in Iran. *Am.-Eurasian J. Toxicol. Sci.* **2011**, *3*, 190–193. [CrossRef]
36. Ku, S. Finding and Producing Probiotic Glycosylases for the Biocatalysis of Ginsenosides: A Mini Review. *Molecules* **2016**, *21*, 645. [CrossRef] [PubMed]
37. Biji, K.B.; Ravishankar, C.N.; Venkateswarlu, R.; Mohan, C.O.; Srinivasa Gopal, T.K. Biogenic Amines in Seafood: A review. *J. Food Sci. Technol.* **2016**, *53*, 2210–2218. [CrossRef] [PubMed]
38. Jansen, S.C.; van Dusseldorp, M.; Bottema, K.C.; Dubois, A.E. Intolerance to Dietary Biogenic Amines: A Review. *Ann. Allergy Asthma Immunol.* **2003**, *91*, 233–240. [CrossRef]
39. Burdychová, R.; Komprda, T. Biogenic Amine-Forming Microbial Communities in Cheese. *FEMS Microbiol. Lett.* **2007**, *276*, 149–155. [CrossRef] [PubMed]
40. Priyadarshani, W.M.D.; Rakshit, S.K. Screening Selected Strains of Probiotic Lactic Acid Bbacteria for Their Ability to Produce Biogenic Amines (Histamine and Tyramine). *Int. J. Food. Sci. Technol.* **2011**, *46*, 2062–2069. [CrossRef]
41. Lorencová, E.; Buňková, L.; Matoulková, D.; Dráb, V.; Pleva, P.; Kubáň, V.; Buňká, F. Production of Biogenic Amines by Lactic Acid Bacteria and Bifidobacteria Isolated from Dairy Products and Beer. *Int. J. Food. Sci. Technol.* **2012**, *47*, 2086–2091. [CrossRef]
42. Kalač, P.; Švecová, S.; Pelikánová, T. Levels of Biogenic Amines in Typical Vegetable Products. *Food Chem.* **2002**, *77*, 349–351. [CrossRef]
43. Ten Brink, B.; Damink, C.; Joosten, H.M.L.J.; Huis in't Veld, J.H.J. Occurrence and Formation of Biologically Active Amines in Foods. *Int. J. Food Microbiol.* **1990**, *11*, 73–84. [CrossRef]
44. Manca, G.; Porcu, A.; Ru, A.; Salaris, M.; Franco, M.A.; De Santis, E.P.L. Comparison of γ-Aminobutyric Acid and Biogenic Amine Content of Different Types of Ewe's Milk Cheese Produced in Sardinia. *Ital. J. Food Saf.* **2015**, *4*, 123–128. [CrossRef] [PubMed]
45. Pollack, P.F.; Koldovskỳ, O.; Nishioka, K. Polyamines in Human and Rat Milk and in the Infant Formulas. *Am. J. Clin. Nutr.* **1992**, *56*, 371–375. [CrossRef] [PubMed]
46. EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on Risk Based Control of Biogenic Amine Formation in Fermented Foods. *EFSA J.* **2011**, *9*, 2393. [CrossRef]
47. Borriello, S.P.; Hammes, W.P.; Holzapfel, W.; Marteau, P.; Schrezenmeir, J.; Vaara, M.; Valtonen, V. Safety of Probiotics That Contain Lactobacilli or Bifidobacteria. *Clin. Infect. Dis.* **2003**, *36*, 775–780. [CrossRef] [PubMed]
48. Ammor, M.S.; Flórez, A.B.; Mayo, B. Antibiotic Resistance in Non-Enterococcal Lactic Acid Bacteria and Bifidobacteria. *Food Microbiol.* **2007**, *24*, 559–570. [CrossRef] [PubMed]
49. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Guidance on the Assessment of Bacterial Susceptibility to Antimicrobials of Human and Veterinary Importance. *EFSA J.* **2012**, *10*, 2740. [CrossRef]
50. Flórez, A.B.; Ammor, M.S.; Mayo, B.; van Hoek, A.H.A.M.; Aarts, H.J.M.; Huys, G. Antimicrobial Susceptibility Profiles of 32 Type Strains of *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus* spp. *Int. J. Antimicrob. Agents* **2008**, *31*, 484–486. [CrossRef] [PubMed]

51. Georgieva, R.; Yochevab, L.; Tserovskab, L.; Zhelezovab, G.; Stefanovaa, N.; Atanasovaa, A.; Dangulevaa, A.; Ivanovaa, G.; Karapetkova, N.; Rumya, N.; et al. Antimicrobial Activity and Antibiotic Susceptibility of *Lactobacillus* and *Bifidobacterium* spp. Intended for Use as Starter and Probiotic Cultures. *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 84–91. [CrossRef] [PubMed]
52. Duranti, S.; Lugli, G.A.; Mancabelli, L.; Turroni, F.; Milani, C.; Mangifesta, M.; Ferrario, C.; Anzalone, R.; Viappiani, A.; van Sinderen, D.; et al. Prevalence of Antibiotic Resistance Genes among Human Gut-Derived Bifidobacteria. *Appl. Environ. Microbiol.* **2017**, *83*, e02894-16. [CrossRef] [PubMed]
53. GRAS notice (GRN), No. 685 Generally Recognized as Safe (GRAS) Determination for the Use of Lactobacillus plantarum 299v in Conventional Foods. Available online: https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm544492.pdf (accessed on 20 November 2017).
54. Mättö, J.; van Hoek, A.H.A.M.; Domig, K.J.; Saarela, M.; Floréz, A.B.; Brockmann, E.; Amtmann, E.; Mayo, B.; Aarts, H.J.M.; Danielsen, M. Susceptibility of Human and Probiotic *Bifidobacterium* spp. to Selected Antibiotics as Determined by the Etest Method. *Int. Dairy J.* **2007**, *17*, 1123–1131. [CrossRef]
55. Lim, K.S.; Huh, C.S.; Baek, Y.J.A. Selective Enumeration Medium for Bifidobacteria in Fermented Dairy Products. *J. Dairy Sci.* **1995**, *78*, 2018–2112. [CrossRef]
56. Food Code (2018-8). 7. General Test Methods: 4.4.1 Medium 25) TOS-MUP Medium; 4.9.2 Acid-Bacterial Streptococcus and Bifidus. Ministry of Food and Drug Safety in Korea. 2018. Available online: http://www.foodsafetykorea.go.kr/portal/safefoodlife/food/foodRvlv/foodRvlv.do (accessed on 5 April 2018).
57. National Food Safety Standard. *Microbiological Examination of Food—Examination of Lactic Acid Bacteria (GB4789.35-2016) 4. Medium and Reagents 4.2 Medium and Reagents*; China Food and Drug Ministration: Beijing, China, 2016.
58. Agency for Healthcare Research and Quality Advancing Excellence in Health Care (AHRQ). *Safety of Probiotics to Reduce Risk and Prevent or Treat Disease*; Southern California Evidence-Based Practice Center: Santa Monica, CA, USA, 2011.
59. Fouhy, F.; Motherway, M.O.; Fitzgerald, G.F.; Ross, R.P.; Stanton, C.; van Sinderen, D.; Cotter, P.D. In Silico Assig ned Resistance Genes Confer *Bifidobacterium* with Partial Resistance to Aminoglycosides but Not to β-Lactams. *PLoS ONE* **2013**, *8*, e82653. [CrossRef] [PubMed]
60. Gueimonde, M.; Sánchez, B.; Reyes-Gavilán, C.G.; Margolles, A. Antibiotic Resistance in Probiotic Bacteria. *Front. Microbiol.* **2013**, *18*, 202. [CrossRef] [PubMed]
61. UniProt. UniProtKB-O52836 (TETW_BUTFI). Available online: http://www.uniprot.org/uniprot/O52836 (accessed on 28 March 2018).
62. De Groote, M.A.; Frank, D.N.; Dowell, E.; Glode, M.P.; Pace, N.R. *Lactobacillus rhamnosus* GG Bacteremia Associated with Probiotic Use in a Child with Short Gut Syndrome. *Pediatr. Infect. Dis. J.* **2005**, *24*, 278–280. [CrossRef] [PubMed]
63. Liong, M.T. Safety of Probiotics: Translocation and Infection. *Nutr. Rev.* **2008**, *66*, 192–202. [CrossRef] [PubMed]
64. Ruas-Madiedo, P.; Gueimonde, M.; Fernández-García, M.; de los Reyes-Gavilán, C.G.; Margolles, A. Mucin Degradation by *Bifidobacterium* Strains Isolated from the Human Intestinal Microbiota. *Appl. Environ. Microbiol.* **2008**, *74*, 1936–1940. [CrossRef] [PubMed]
65. Ruseler-van Embden, J.G.; Liesholt, L.M.; Gosselink, M.J.; Marteau, P. Inability of *Lactobacillus casei* Strain GG, *L. acidophilus* and *Bifidobacterium bifidum* to Degrade Intestinal Mucus Glycoproteins. *Scand. J. Gastroenterol.* **1995**, *30*, 675–680. [CrossRef] [PubMed]
66. Abe, F.; Muto, M.; Yaeshima, T.; Iwatsuki, K.; Aihara, H.; Ohashi, Y.; Fujisawa, T. Safety Evaluation of Probiotic Bifidobacteria by Analysis of Mucin Degradation Activity and Translocation Ability. *Anaerobe* **2010**, *16*, 131–136. [CrossRef] [PubMed]
67. Chaney, A.L.; Marbach, E.P. Modified Reagents for Determination of Urea and Ammonia. *Clin. Chem.* **1962**, *8*, 130–132. [PubMed]
68. Kim, N.Y.; Ji, G.E. Characterization of the Production of Biogenic Amines and Gamma-Aminobutyric Acid in the Soybean Pastes Fermented by *Aspergillus oryzae* and *Lactobacillus brevis*. *J. Microbiol. Biotechnol.* **2015**, *25*, 464–468. [CrossRef] [PubMed]
69. International Organization for Standardization (ISO). *Milk and Milk Products-Determination of the Minimal Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-Enterococcal Lactic Acid Bacteria (LAB)*; ISO 10932:2010 (IDF 223:2010); ISO: Geneve, Switzerland, 2010.

70. Tannock, G.W. Conjugal Transfer of Plasmid pAMßi in *Lactobacillus reuteri* and between Lactobacilli and *Enterococcus faecalis*. *Appl. Environ. Microbiol.* **1987**, *53*, 2693–2695. [PubMed]
71. Guo, H.L.; Pan, L.; Li, L.N.; Lu, J.; Kwok, L.; Menghe, B.; Zhang, H.P.; Zhang, W.Y. Characterization of Antibiotic Resistance Genes from *Lactobacillus* Isolated from Traditional Dairy Products. *J. Food Sci.* **2017**, *82*, 724–730. [CrossRef] [PubMed]
72. Thapa, D.; Louis, P.; Losa, R.; Zweifel, B.; Wallace, R.J. Essential oils have different effects on human pathogenic and commensal bacteria in mixed faecal fermentations compared with pure cultures. *Microbiology.* **2015**, *161*, 441–449. [CrossRef] [PubMed]
73. Rojo-Bezares, B.; Saenz, Y.; Poeta, P.; Zarazaga, M.; Ruiz-Larrea, F.; Torres, C. Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. *Int. J. Food Microbiol.* **2006**, *111*, 234–240. [CrossRef] [PubMed]
74. Ouoba, L.I.; Lei, V.; Jensen, L.B. Resistance of potential probiotic lactic acid bacteria and bifi-dobacteria of African and European origin to antimicrobials: Determination and transferability of the resistance genes to other bacteria. *Int. J. Food Microbiol.* **2008**, *121*, 217–224. [CrossRef] [PubMed]
75. Liu, C.; Zhang, Z.Y.; Dong, K.; Yuan, J.P.; Guo, X.K. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. *Biomed Environ Sci.* **2009**, *22*, 401–412. [CrossRef]
76. Aquilanti, L.; Garofalo, C.; Osimani, A.; Silvestri, G.; Vignaroli, C.; Clementi, F. Isolation and molecular characterization of antibiotic-resistant lactic acid bacteria from poultry and swine meat products. *J. Food. Prot.* **2007**, *70*, 557–565. [CrossRef] [PubMed]
77. Gad, G.F.; Abdel-Hamid, A.M.; Farag, Z.S. Antibiotic resistance in lactic acid bacteria isolated from some pharmaceutical and dairy products. *Braz. J. Microbiol.* **2014**, *45*, 25–33. [CrossRef] [PubMed]
78. Charpentier, E.; Gerbaud, G.; Courvalin, P. Characterization of a new class of tetracycline-resistance gene tet(S) in *Listeria monocytogenes* BM4210. *Gene* **1993**, *131*, 27–34. [CrossRef]
79. Mellmann, A.; Andersen, P.S.; Bletz, S.; Friedrich, A.W.; Kohl, T.A.; Lilje, B.; Niemann, S.; Prior, K.; Rossen, J.W.; Harmsen, D. High Interlaboratory Reproducibility and Accuracy of Next-Generation-Sequencing-Based Bacterial Genotyping in a Ring Trial. *J. Clin. Microbiol.* **2017**, *55*, 908–913. [CrossRef] [PubMed]
80. Joensen, K.G.; Scheutz, F.; Lund, O.; Hasman, H.; Kaas, R.S.; Nielsen, E.M.; Aarestrup, F.M. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Micobiol.* **2014**, *52*, 1501–1510. [CrossRef] [PubMed]

The long-term effects of probiotics in the therapy of ulcerative colitis: A clinical study

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Aim. Intestinal dysbiosis seems to be the leading cause of inflammatory bowel diseases, and probiotics seems to represent the proper support against their occurrence. Actually, probiotic blends and anti-inflammatory drugs represent a weapon against inflammatory bowel diseases. The present study evaluates the long-term (2 years) effects of combination therapy (mesalazine plus a probiotic blend of Lactobacillus salivarius, Lactobacillus acidophilus and Bifidobacterium bifidus strain BGN4) on ulcerative colitis activity.
Method. Sixty patients with moderate-to-severe ulcerative colitis were enrolled: 30 of them were treated with a single daily oral administration of mesalazine 1200 mg; 30 patients received a single daily oral administration of mesalazine 1200 mg and a double daily administration of a probiotic blend of Lactobacillus salivarius, Lactobacillus acidophilus and Bifidobacterium bifidus strain BGN4. The treatment was carried out for two years and the clinical response evaluated according to the Modified Mayo Disease Activity Index.
Results. All patients treated with combination therapy showed better improvement compared to the controls. In particular, the beneficial effects of probiotics were evident even after two years of treatment.
Conclusions. A long-term treatment modality of anti-inflammatory drugs and probiotics is viable and could be an alternative to corticosteroids in mild-to moderate ulcerative colitis.

Key words: bifidobacteria, inflammatory bowel diseases, lactobacilli, microbiota, ulcerative colitis

Received: January 18, 2016; Accepted with revision: August 10, 2016; Available online: September 13, 2016
http://dx.doi.org/10.5507/bp.2016.044

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INTRODUCTION

Inflammatory bowel disease worldwide incidence and prevalence have been increasing in the last few decades. Ulcerative colitis (UC) is one of the two major types of IBD, along with Crohn disease (CD). Unlike CD, which can affect any part of the gastrointestinal tract, UC characteristically involves the large bowel[1,2].

There is a large debate about the exact aetiology of UC. Proposed causes include environmental factors, immune dysfunction, and a likely genetic predisposition. The current hypothesis is that genetically susceptible individuals have abnormalities of humoral and cell-mediated immunity and a generalized enhanced reactivity against commensal intestinal bacteria[3]. This dysregulated mucosal immune response predisposes to colonic inflammation[3]. Whether these abnormalities are the cause or the result of the intense systemic inflammatory response in UC is still unresolved. However, it is well documented that bacterial microflora is altered in patients with active disease[4]. Recent studies reported a great variation in the effects of microbiota, focusing, in particular, on the effects of a pro-inflammatory enterotype on mucosal layer and disease activity[5].

Modulation of the intestinal microbiota can be performed either by antibiotics or by probiotics, but the former are not good candidates for chronic disease because of antibiotic resistance, potential side effects, and ecological concerns[6]. Therefore, the use of probiotics in IBD could be considered a potential aid to the current conventional therapies. An accurate analysis of scientific data proves that the efficacy of probiotics in the treatment of various diseases has been amply demonstrated and confirmed[7-9]. Actually, several studies have focused on the effects of probiotic blends on enteral microbiota, especially in those cases of dysbiosis, when the normal concentration of "good" bacterial flora is impaired by the presence of pathogenic bacteria[10,11].

Currently, the standard treatment of UC relies on an initial medical management with corticosteroids and anti-inflammatory agents, such as mesalazine, in conjunction with a symptomatic treatment with antidiarrheal agents and rehydration. These treatments have been proven not to be always reliable in controlling the clinical course of

Table 1. Modified Mayo Disease Activity Index.

Grade	Bowel frequency	Rectal bleeding	Physician's global assessment	Endoscopy/sigmoidoscopy finding
0	Normal number of stools per day for this patient	No blood seen	Normal	Normal or inactive disease
1	1 or 2 more stools than normal	Streaks of blood with stool less than half the time	Mild disease	Mild disease (erythema, decreased vascular pattern)
2	3 or 4 more stools than normal	Obvious blood with stool most of the time	Moderate disease	Moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3	5 or more stools than normal	Blood alone passed	Severe disease	Severe disease (spontaneous bleeding, ulceration)

the disease[12,13] and present side effects in a significant proportion of patients who do not tolerate the existing treatments[14]. In the present open-labeled randomized controlled study, standard corticosteroid treatment was substituted for a combination therapy (anti-inflammatory + probiotics) for two years, in moderate-to-severe UC and the disease activity was periodically followed-up according to the Modified Mayo Disease Activity Index (MMDAI) (ref.[15]).

Table 2. Modified Mayo Disease Activity Index (MMDAI) from t_0 to t_4, in group A, B and A vs B.

MMDAI	A	B	A vs B
t_0	10.1±1.4	10.2±1.6	ns
t_1	7.2±2.0	6.0±1.5	$P = 0.0109$
t_2	5.4±1.3	4.8±0.7	$P = 0.0232$
t_3	5.7±1.4	4.8±0.8	$P = 0.0035$
t_4	6.1±2.0	4.4±0.8	$P = 0.0001$

Table 3. Physician's global assessment from t_0 to t_4, in group A, B and A vs B.

Physician's global assessment	A	B	A vs B
t_0	2.6±0.7	2.7±0.5	ns
t_1	1.9±0.7	1.7±0.7	ns
t_2	1.5±0.6	1.3±0.5	ns
t_3	1.4±0.6	1.2±0.4	ns
t_4	1.5±0.6	1.2±0.4	$P = 0.0040$

MATERIALS AND METHODS

From January 2011 to December 2012, 60 UC patients were evaluated clinically and endoscopically to establish their disease activity, in accordance with the MMDAI (ref.[15]), a simplified composite score incorporating four variables: stool frequency, rectal bleeding, mucosal appearance and physician's rating of disease activity (Table 1). By employing a four point scoring scale for each variable, the relative simplicity of the index reduces the impact of physician and patient subjectivity in disease scoring. Interestingly, the stool frequency score is not an absolute number, but relative to "normal" for that subject. The MMDAI was modified by the deletion of "friability" from an endoscopy score equal to 1; in fact, the assessment of mucosa "friability" is an important subjective parameter and its deletion contributes to the objectivity of the chosen score. Patients over 18 years of age with UC and a moderate-to-severe disease (activity index: 8-12) were considered eligible for the study. The diagnosis of UC, was established on the basis of standard clinical, endoscopic and histological criteria. All subjects were out-patients, attending our Gastroenterology Unit. A condition of steroid dependence, renal impairment, pregnancy, lactation or established low compliance, was considered as an exclusion criterium. The use of other drugs, such as rectal mesalazine or steroid preparations, was not allowed during investigation. Patients were free to leave the study at any time (withdrawal of consent). Other reasons for withdrawal from the investigation were: lack of adherence to the therapeutic schedule or programmed controls (< 85%, poor compliance); onset of symptoms of relapse, confirmed by instrumental procedures (therapeutic failure); onset of drug-related adverse events requiring interruption of treatment. The study was approved by the local ethics committee and all participants subscribed to an informed consent.

The patients were divided into two homogeneous groups: group A, including 22 male and 8 female subjects aged 35-69 years (mean 43 years), was treated pharmacologically with 1200 mg of oral mesalazine once-daily (Mesavancol® 1200 mg cpr, Giuliani spa, Milan); group B, which included 19 male and 11 female UC patients aged between 28 and 71 years (mean 46 years), was treated with a single daily administration of oral mesalazine 1200 mg (Mesavancol® 1200 mg cpr, Giuliani spa, Milan) and a double administration of a probiotic blend of Lactobacillus salivarius, Lactobacillus acidophilus and Bifidobacterium bifidus strain BGN4 (Acronelle®, Bromatech srl, Milan, Italy). The treatment was carried out in both groups for two years.

Considering as "t_0" the first evaluation, all patients were subsequently followed-up for 6 (t_1), 12 (t_2), 18 (t_3) and 24 (t_4) months. A new reassessment of the activity score was carried out at every check-point. Data were evaluated statistically using the ANOVA method for re-

peated measures (ANOVA Repeated Measures), and the two groups compared by means of t test (unpaired t test).

RESULTS

During the 24 months of study, patients treated with mesalazine and probiotic blend showed better results than those reached by patients treated with mesalazine alone. In particular, as Table 2 suggests, the benefits of the probiotic blend in combination with the anti-inflammatory treatment are tangible and statistically significant after a period of at least 18 months. Both groups showed an effective improvement of patient general clinical condition which is reflected by a global improvement of their MMDAI, but group B patients advantaged from the use of probiotics which, probably, enhanced the effects of the anti-inflammatory treatment.

Table 3 underlines the positive effects of single daily administration of mesalazine in UC patients. Interestingly, in group B, the anti-inflammatory action of the treatment is evidently powered by probiotics, which ameliorate the clinical response and shorten significantly the time of recovery. Furthermore, the combined therapy contributed to maintain constantly low the score in group B, whose patients showed an acceptable overall clinical condition up to the end of the study. However, a slight deterioration could be observed over time, in group A. Data analysis during the 2 years of treatment in both groups, corroborates the showed results, remarking the slight benefit of the combination therapy compared to the sole use of mesalazine (Table 3).

Stool frequency, the second parameter considered, also showed a significant improvement due to the combined treatment, with a slight reduction of frequency in group B compared to group A (Table 4). Strangely, there was a slight deterioration at 18 months in group B, whereas in group A, even in this case, a moderate loss of effectiveness of the anti-inflammatory therapy could be recognized over time. Comparing the two groups, the reduction of stool frequency became statistically significant in group B, at 6 and 24 months of therapy. The table shows a small reversal of the trend at 18 months, when the patients of group B seemed to lose the advantage supplied by probiotics.

Considering the endoscopic picture (Table 5), both treatments were already effective only after 6 months. In group B, patients showed a significant improvement of intestinal mucosa aspect, compared to group A, and, as already remarked for the overall clinical condition, the beneficial effects of combination therapy remained constant for the entire period of study, compared to group A, whose patients lost some of those benefits already at t_3.

The evaluation of rectal bleeding more or less followed the same time lapse already seen for the endoscopic picture (Table 6). In fact, bleeding decreased in both groups already at t_1, but whereas group B enjoyed the positive effects of probiotics up to 2 years, showing a certain constancy of values at each check-point, group A, once again, got progressively worse from 18 months of treatment onwards.

Table 4. Stool frequency from t_0 to t_4, in group A, B and A vs B.

Stool frequency	A	B	A vs B
t_0	2.4±0.5	2.4±0.6	ns
t_1	1.8±0.6	1.4±0.5	$P = 0.0021$
t_2	1.3±0.5	1.2±0.4	ns
t_3	1.3±0.5	1.3±0.5	ns
t_4	1.5±0.6	1.0±0.2	$P = 0.0006$

Table 5. Endoscopic picture from t_0 to t_4, in group A, B and A vs B.

Endoscopic finding	A	B	A vs B
t_0	2.5±0.5	2.5±0.6	ns
t_1	1.8±0.8	1.5±0.6	ns
t_2	1.3±0.5	1.2±0.4	ns
t_3	1.6±0.5	1.1±0.3	$P = 0.0005$
t_4	1.6±0.6	1.1±0.3	$P = 0.0005$

Table 6. Rectal bleeding from t_0 to t_4, in group A, B and A vs B.

Rectal bleeding	A	B	A vs B
t_0	2.6±0.6	2.5±0.6	ns
t_1	1.7±0.7	1.4±0.5	$P = 0.0498$
t_2	1.3±0.4	1.1±0.3	ns
t_3	1.4±0.5	1.1±0.3	$P = 0.0374$
t_4	1.5±0.7	1.1±0.3	$P = 0.0024$

DISCUSSION

In the last few years, intestinal microbiota seems to be increasingly involved in UC pathogenesis[16,17]. The characteristic chronic inflammation of the colonic mucosa in UC is likely due to the constant exposure of the mucosal layer to antigenic endoluminal stimuli. Numerous studies have identified in intestinal dismicrobism the most important endoluminal antigenic stimulus; this, along with the change of the whole intestinal microenvironment, could hyperstimulate the immune system and trigger the inflammatory process[18-20].

Microbiological studies have detected a significant reduction of Bifidobacteria and Lactobacilli and an overgrowth of specific pathogenic strains, such as Deltaproteobacteria and Bilophila wadsworthia. The use of probiotics in combination with the standard treatment, improves patients' quality of life and life expectancy, reducing significantly clinical symptoms and minimizing side effects[21-25]. The results of our study confirm the beneficial effects of probiotics on UC activity, partly by improving patient's response to anti-inflammatory treatment. Comparing patients treated with probiotic blend and mesalazine, and those patients who received the anti-

inflammatory alone, data report a significant improvement of MMDAI in the former group, after 18 months. Interestingly, the association of probiotics with mesalazine, seems to guarantee a stable effect in the whole period of observation, different from the anti-inflammatory therapy alone, which seems to be burdened by an unsteady response. Oral 5-ASA administered once daily has been demonstrated to be as effective and safe as conventional dosing (twice or three times daily) for maintenance of remission in quiescent UC (ref.[26]). The choice of testing a specific probiotic blend (Acronelle®, Bromatech srl, Milan, Italy) was suggested by the distinguishing features of the bacterial strains contained in the considered probiotic blend: Lactobacillus salivarius has a high anti-inflammatory and antibacterial activity, especially against some of those pathogenic bacterial strains, usually present on the intestinal mucosa of IBD patients[27]; Bifidobacterium bifidum BGN 4 supports Lactobacilli against inflammation, thanks to the chiro-inositol present in its cell membrane, which seems to reduce the production of pro-inflammatory cytokines[28]. Although guidelines[29] suggest to treat moderate-to-severe UC with corticosteroids, the contemporary use of Mesavancol® and Acronelle® allowed to reach an encouraging result, avoiding all those therapy side effects which usually decrease patient compliance. For the first time, the long-term efficacy (2 years) of such a probiotic blend has been proven, also demonstrating the synergistic effect on disease activity, especially when coupled with anti-inflammatory treatment. Several authors have already contributed to research in this field, however, none of them has followed-up moderate-to-severe UC patients, for a such long period of time. Ishikawa et al. (ref.[30]) reported that Bifidobacteria-fermented milk (BFM) supplementation reduces the luminal butyrate concentration, a key molecule in the remission of colitis. This reduction reflects the increased uptake or oxidation of SCFAs by the improved colorectal mucosa. Similarly, Kato et al. (ref.[31]) found increased levels of faecal butyrate, propionate, and SCFA acid concentrations in patients with active UC (mild to moderate), who received BFM together with conventional treatment. In this pilot study, patients supplemented with BFM showed a significantly lower clinical activity index than the placebo group. Likewise, the post-treatment endoscopic index and histological score were reduced in the BFM group. Probio-Tec AB-25, a mixture of Lactobacillus acidophilus strain La-5 and Bifidobacterium animalis subsp. lactis strain Bb-12, was tested for the maintenance of remission in patients with left-sided UC, in a 1-year, prospective, randomized, double-blind and placebo controlled trial[32]. The safety and tolerance of Probio-Tec AB-25 and the placebo were good. Gastrointestinal symptoms were reported equally in both treatment groups and a relationship between Probio-Tec 25 and gastrointestinal side effects could not be established. At weeks 4 and 28, Bb-12 or La-5 were detected in 11 patients receiving probiotics. Five patients in the probiotic group (25%) and one patient in the placebo group (8%) maintained remission after 1 year of treatment. In the probiotic group, the median time to relapse was 125.5 days, versus 104 days in the placebo group. The use of BIFICO (oral capsules of live enterococci, bifidobacteria, and lactobacilli) in combination with sulphasalazine and glucocorticoid exerts some beneficial effects in preventing the relapse of UC (ref.[33]). The administration of BIFICO plus sulphasalazine and glucocorticoid to UC patients enlarged the number of bifidobacteria and lactobacilli and reduced the number of enterococci, bacteroides, and bifidobacteria present in the faeces compared with the control group. The most studied probiotic in clinical trials is Lactobacillus rhamnosus, which is present in the bowel of healthy individuals. Zocco et al. (ref.[34]) studied the efficacy of Lactobacillus rhamnosus GG (LGG) supplementation versus standard mesalazine for maintaining disease remission in UC patients. After 6 and 12 months of treatment, the percentage of patients maintaining clinical remission was, respectively, 91% and 85% for the LGG group (1.8 × 1010 viable bacteria/day), 87% and 80% for the mesalazine group (2400 mg/day), and 94% and 84% for the combined treatment (LGG plus mesalazine). The oral administration of Lacteol (Lacteol Fort, Rameda, Egypt), a probiotic preparation that contains 1 × 1010 CFU of Lactobacillus delbrueckii and Lactobacillus fermentum, together with 2400 mg/day of sulfasalazine, during 8 weeks, to UC patients with chronic diarrhea, inhibited the extent of inflammation, prevented mucosal injury, and alleviated colitis[35]. In children with distal active UC, rectal administration of Lactobacillus reuteri ATCC 55730 (as an enema solution containing 1 × 1010 CFU) for 8 weeks in addition to standard oral mesalazine, resulted in a significant decrease in the MDAI compared with the children that received the corresponding placebo. In addition, all of the children on Lactobacillus reuteri had a clinical response, whereas only 53% of the children on the placebo responded. Clinical remission was achieved in 31% of the Lactobacillus reuteri group and in no children of the placebo group[36]. D'Incà et al. (ref.[37]) evaluated the effect of an 8-week oral and/or rectal administration of Lactobacillus casei DG on colonic-associated microbiota, mucosal cytokine balance, and TLR expression in patients with mild left-sided UC. The patients were divided into three groups: the first group received oral 5-ASA alone, the second group received oral 5-ASA plus oral Lactobacillus casei DG (8 × 108 CFU), and the third group received oral 5-ASA and rectal Lactobacillus casei DG (8 × 108 CFU). A significant improvement of the histological scores was found in patients receiving the probiotic strain by the oral or rectal route of administration. Nevertheless, oral supplementation with Lactobacillus casei DG did not have a significant effect on the counts of Enterobacteriaceae or Lactobacillus. However, the occurrence of Lactobacillus and Enterobacteriaceae cultured from biopsy specimens was increased and decreased, respectively, in the group that took the probiotic rectally. Moreover, the rectal administration of Lactobacillus casei DG significantly reduced TLR-4 and IL-1β levels and significantly increased mucosal IL-10.

Probiotics act into the inflamed intestine, destroying "bad" bacterial flora and restoring previous micro-environment conditions. Probably, such an effect this prevents that abnormal reaction of the human immune system at

the base of intestinal autoimmune diseases. In this case, patients treated with probiotics showed an overall improvement of all studied parameters: patients showed a better clinical response, reduced significantly their stool frequency, maintained easily their haemoglobin values and exhibited a significant improvement of their gut mucosa condition. Likely, a random choice of probiotic strains should not be the right way to cure IBD definitely, but the present study demonstrates the efficacy of some "good" bacterial strains in assisting anti-inflammatory drug mechanism of action.

Author contributions: VDP: study design, results analysis; MR, AILM, GT: study design, final approval; AMG, FC, PD: results analysis; GD, SB: literature search.
Conflict of interest statement: None declared.

REFERENCES

1. Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 2012;142:46-54.
2. Cotran RS, Collins T, Robbins SL, Kumar V. Pathologic Basis of Disease, 3rd edn. Philadelphia: WB Saunders; 1998.
3. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature 2007;448:427-34.
4. Almeida MG, Kiss DR, Zilberstein B, Quintanilha AG, Teixeira MG, Habr-Gama A. Intestinal mucosa-associated microflora in ulcerative colitis patients before and after restorative proctocolectomy with an ileoanal pouch. Dis Colon Rectum 2008;51:1113-9.
5. Hildebrand F, Nguyen TL, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, Liston A, Raes J. Inflammation-associated enterotypes, host genotype cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol 2013;14:R4.
6. Tursi A, Brandimarte G, Papa A, Giglio A, Elisei W, Giorgetti GM, Forti G, Morini S, Hassan C, Pistoia MA, Modeo ME, Rodino' S, D'Amico T, Sebkova L, Sacca' N, Di Giulio E, Luzza F, Imeneo M, Larussa T, Di Rosa S, Annese V, Danese S, Gasbarrini A. Treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment: a double blind, randomized, placebo-controlled study. Am J Gastroenterol 2010;105:2218-27.
7. Tomasello G, Palumbo VD, Miceli A, Sinagra E, Bruno A, Abruzzo A, Cappello F, Patti AM, Giglio RM, Damiani P, Tomasello R, Noto M, Arculeo VM, Accardo MF, Lo Monte AI. Probiotics and conventional therapy: new frontiers in therapeutic approach in articular manifestations of IBD. Progr Nutr 2014;16:176-87.
8. Sinagra E, Tomasello G, Cappello F, Leone A, Cottone M, Bellavia M, Rossi F, Facella T, Damiani P, Zeenny MN, Damiani F, Abruzzo A, Damiano G, Palumbo VD, Cocchi M, Jurjus A, Spinelli G, Lo Monte AI, Raimondo D. Probiotics, prebiotics and synbiotics in inflammatory bowel diseases: state-of-the-art and new insights. J Biol Regul Homeost Agents 2013;27:919-33.
9. Kvasnovsky CL, Bjarnason I, Papagrigoriadis S. What colorectal surgeons should know about probiotics: a review. Colorectal Dis 2015;17:840-8.
10. Mack DR. Probiotics in inflammatory bowel diseases and associated conditions. Nutrients 2011;3:245-64.
11. D'Haens GR, Sartor RB, Silverberg MS, Petersson J, Rutgeerts P. Future directions in inflammatory bowel disease management. J Crohns Colitis 2014;8:726-34.
12. Langan RC, Gotsch PB, Krafczyk MA, Skillinge DD. Ulcerative colitis: diagnosis and treatment. Am Fam Physician 2007;76:1323-30.
13. MacDermott RP, Green JA, Ashley CC. What is the optimal therapy for severe ulcerative colitis? Inflamm Bowel Dis 2008;14:S228-31.
14. Miele E, Pascarella F, Giannetti E, Quaglietta L, Baldassano RN, Staiano A. Effect of a probiotic preparation (VSL#3) on induction and maintenance of remission in children with ulcerative colitis. Am J Gastroenterol 2009;104:437-43.
15. Scherl EJ, Pruitt R, Gordon GL, Lamet M, Shaw A, Huang S, Mareya S, Forbes WP. Safety and efficacy of a new 3.3 g b.i.d. tablet formulation in patients with mild-to-moderately-active ulcerative colitis: a multicenter, randomized, double-blind, placebo-controlled study. Am J Gastroenterol 2009;104:1452-9.
16. Bellavia M, Damiano G, Gioviale MC, Palumbo VD, Cacciabaudo F, Buscemi G, Lo Monte AI. Abnormal expansion of segmented filamentous bacteria in the gut: a role in pathogenesis of chronic inflammatory intestinal diseases? Rev Microbiol 2011;22:45-7.
17. Tomasello G, Bellavia M, Palumbo VD, Gioviale MC, Damiani P, Lo Monte AI . From gut microflora imbalance to mycobacteria infection: is there a relationship with chronic intestinal inflammatory diseases? Ann Ital Chir 2011;82:361-8.
18. Rodolico V, Tomasello G, Zerilli M, Martorana A, Pitruzzella A, Gammazza AM, David S, Zummo G, Damiani P, Accomando S, Conway de Macario E, Macario AJ, Cappello F. Hsp60 and Hsp10 increase in colon mucosa of Crohn's disease and ulcerative colitis. Cell Stress Chaperones 2010;15:877-84.
19. Tomasello G, Rodolico V, Zerilli M, Martorana A, Bucchieri F, Pitruzzella A, Marino Gammazza A, David S, Rappa F, Zummo G, Damiani P, Accomando S, Rizzo M, de Macario EC, Macario AJ, Cappello F. Changes in immunohistochemical levels and subcellular localization after therapy and correlation and colocalization with CD68 suggest a pathogenetic role of Hsp60 in ulcerative colitis. Appl Immunohistochem Mol Morphol 2011;19:552-61.
20. Tomasello G, Sciumé C, Rappa F, Rodolico V, Zerilli M, Martorana A, Cicero G, De Luca R, Damiani P, Accardo FM, Romeo M, Farina F, Bonaventura G, Modica G, Zummo G, Conway de Macario E, Macario AJ, Cappello F. Hsp10, Hsp70, and Hsp90 immunohistochemical levels change in ulcerative colitis after therapy. Eur J Histochem 2011;55:e38.
21. Yoshimatsu Y, Yamada A, Furukawa R, Sono K, Osamura A, Nakamura K, Aoki H, Tsuda Y, Hosoe N, Takada N, Suzuki Y.Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis. World J Gastroenterol 2015;21:5985-94.
22. Chen WX, Ren LH, Shi RH. Enteric micobiota leads to new therapeutic strategies for ulcerative colitis. World J Gastroenterol 2014;20:15657-63.
23. Saez-Lara MJ, Gomez-Llorente C, Plaza-Diaz J, Gil A.The role of probiotic lactic acid bacteria and bifidobacteria in the prevention and treatment of inflammatory bowel disease and other related diseases: a systematic review of randomized human clinical trials. Biomed Res Int 2015;2015:505878.
24. Strisciuglio C, Miele E, Giugliano FP, Vitale S, Andreozzi M, Vitale A, Catania MR, Staiano A, Troncone R, Gianfrani C. Probiotic bacteria enhance antigen sampling and processing by dendritic cells in pediatric Inflammatory Bowel Disease. Dig Liver Dis 2015;21:1491-8.
25. Tralongo P, Tomasello G, Sinagra E, Damiani P, Leone A, Palumbo VD, Giammanco M, Di Majo D, Damiani F, Abruzzo A, Bruno A, Cassata G, Cicero L, Noto M, Tomasello R, Lo Monte AI. The role of butyric acid as a protective agent against inflammatory bowel diseases. EMBJ 2014;9:24-35.
26. Sandborn WJ, Korzenik J, Lashner B, Leighton JA, Mahadevan U, Marion JF, Safdi M, Sninsky CA, Patel RM, Friedenberg KA, Dunnmon P, Ramsey D, Kane S. Once-daily dosing of delayed-release oral mesalamine (400-mg tablet) is as effective as twice-daily dosing for maintenance of remission of ulcerative colitis. Gastroenterology 2010;138:1286-96.
27. Collins JK. Demonstration of functional properties of probiotic lactic acid bacteria. Ind Latte 2001;37:39-61.
28. You HJ, Oh DK, Ji GE. Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from Bifidobacterium bifidum BGN4. FEMS Microbiol Lett 2004;240:131-6.
29. Bressler B, Marshall JK, Bernstein CN, Bitton A, Jones J, Leontiadis GI, Panaccione R, Steinhart AH, Tse F, Feagan B, Toronto Ulcerative Colitis Consensus Group. Clinical practice guidelines for the medical management of nonhospitalized ulcerative colitis: the Toronto consensus. Gastroenterology 2015;148:1035-58.
30. Ishikawa H, Akedo I, Umesaki Y, Tanaka R, Imaoka A, Otani T. Randomized controlled trial of the effect of bifidobacteria-fermented milk on ulcerative colitis. J Am Coll Nutr 2003;22:56-63.
31. Kato K, Mizuno S, Umesaki Y, Ishii Y, Sugitani M, Imaoka A, Otsuka M, Hasunuma O, Kurihara R, Iwasaki A, Arakawa Y. Randomized

placebocontrolled trial assessing the effect of bifidobacteria-fermented milk on active ulcerative colitis. Aliment Pharmacol Ther 2004;20:1133-41.
32. Wildt S, Nordgaard I, Hansen U, Brockmann E, Rumessen JJ. A randomised double-blind placebo-controlled trial with Lactobacillus acidophilus La-5 and Bifidobacterium animalis subsp. lactis BB-12 for maintenance of remission in ulcerative colitis. J Crohns Colitis 2011;5:115-21.
33. Cui HH, Chen CL, Wang JD, Yang YJ, Cun Y, Wu JB, Liu YH, Dan HL, Jian YT, Chen XQ. Effects of probiotic on intestinal mucosa of patients with ulcerative colitis. World J Gastroenterol 2004;10:1521-5.
34. Zocco MA, dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, Novi M, Rigante D, Cazzato IA, Ojetti V, Armuzzi A, Gasbarrini G, Gasbarrini A. Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 2006:23:1567-74.
35. Hegazy SK, El-Bedewy MM. Effect of probiotics on pro-inflammatory cytokines and NF-κB activation in ulcerative colitis. World J Gastroenterol 2010;16:4145-51.
36. Oliva S, Di Nardo G, Ferrari F, Mallardo S, Rossi P, Patrizi G, Cucchiara S, Stronati L. Randomised clinical trial: the effectiveness of Lactobacillus reuteri ATCC 55730 rectal enema in children with active distal ulcerative colitis. Aliment Pharmacol Ther 2012;35:327-34.
37. D'Incà R, Barollo M, Scarpa M, Grillo AR, Brun P, Vettorato MG, Castagliuolo I, Sturniolo GC. Rectal administration of Lactobacillus casei DG modifies flora composition and toll-like receptor expression in colonic mucosa of patients with mild ulcerative colitis. Dig Dis Sci 2011;56:1178-87.

Pediatr Allergy Immunol 2010: 21: e386–e393
DOI: 10.1111/j.1399-3038.2009.00958.x

PEDIATRIC ALLERGY AND IMMUNOLOGY

Effect of probiotic mix (*Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus*) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial

Kim JY, Kwon JH, Ahn SH, Lee SI, Han YS, Choi YO, Lee SY, Ahn KM, Ji GE. Effect of probiotic mix (*Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus*) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial.
Pediatr Allergy Immunol 2010: 21: e386–e393.

Controversy exists regarding the preventive effect of probiotics on the development of eczema or atopic dermatitis. We investigated whether supplementation of probiotics prevents the development of eczema in infants at high risk. In a randomized, double-blind, placebo-controlled trial, 112 pregnant women with a family history of allergic diseases received a once-daily supplement, either a mixture of *Bifidobacterium bifidum* BGN4, *B. lactis* AD011, and *Lactobacillus acidophilus* AD031, or placebo, starting at 4–8 wks before delivery and continuing until 6 months after delivery. Infants were exclusively breast-fed during the first 3 months, and were subsequently fed with breastmilk or cow's milk formula from 4 to 6 months of age. Clinical symptoms of the infants were monitored until 1 yr of age, when the total and specific IgE against common food allergens were measured. A total of 68 infants completed the study. The prevalence of eczema at 1 yr in the probiotic group was significantly lower than in the placebo group (18.2% vs. 40.0%, $p = 0.048$). The cumulative incidence of eczema during the first 12 months was reduced significantly in probiotic group (36.4% vs. 62.9%, $p = 0.029$); however, there was no difference in serum total IgE level or the sensitization against food allergens between the two groups. Prenatal and postnatal supplementation with a mixture of *B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031 is an effective approach in preventing the development of eczema in infants at high risk of allergy during the first year of life.

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Key words: probiotics; *Bifidobacterium; Lactobacillus;* eczema; prevention; infant

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Accepted 11 September 2009

Eczema or atopic dermatitis is a common chronic inflammatory skin disease, mostly occurring in children (1). A recent study has shown that the worldwide prevalence of eczema in childhood was increasing (2), and the prevalence of eczema in Korean children aged 6–12 yrs increased between 1995 (19.7%) and 2000 (27.5%) (3). It is speculated that the increasing prevalence of allergic diseases in developed countries is associated with the so-called 'hygiene hypothesis', in which a lack of infections or other microbial exposures at an early age leads to Th2-dominant immune status and the subsequent development of allergic diseases (4, 5).

Recently, the role of intestinal microflora has been emphasized in the maintenance of normal gut barrier function and development of tolerogenic immune status (6). Mice raised in a germ-free environment failed to develop oral tolerance and had a persistent Th2-dependent immune response, while reconstitution of intestinal microbes during the neonatal period could reverse this immune deviation (7). Infants with allergic diseases showed less intestinal colonization by *Lactobacillus* or *Bifidobacterium* and more colonization by *Clostridium* relative to non-allergic infants (8–10). It is also known that specific intestinal bacteria bring about immune tolerance via the up-regulation of inhibitory Toll-interacting protein (11). These findings suggest that certain gut microbes modulate regulatory T cells, leading to the suppression of allergic disorders (12).

It remains a matter of controversy whether the modification of intestinal microflora by supplementation with probiotic bacteria in early life is effective in preventing eczema. Prenatal and postnatal supplementation with *Lactobacillus rhamnosus* GG has been shown to reduce the prevalence of atopic eczema at 2 yrs of age. This protective effect even continued during 7 yrs of follow-up (13, 14); however, it did not affect the sensitization rate or the development of asthma or allergic rhinitis. In another study, early supplementation with *Lactobacillus acidophilus* (LAVRI-A1) did not reduce the prevalence and severity of atopic dermatitis, but resulted in an increased proportion of infants with allergic sensitization at 1 yr of age (15). Those contradictory findings imply that further studies are needed to assess whether supplementation with probiotic bacteria at an early age has a preventive effect on eczema.

In the present study, we investigated whether prenatal and postnatal administration of a mixture of *Bifidobacteria* and *Lactobacillus* could prevent the development of eczema and sensitization against common food allergens in infants at high risk of atopic disease.

Materials and methods

Study design and participants

This study was a randomized, double-blind, placebo-controlled trial designed to evaluate the preventive effect of probiotics on the development of eczema. A total of 112 pregnant women with a family history of allergic diseases were recruited at Samsung Medical Center (Irwon-dong, Gangnam-gu, Seoul, Korea) from January 2005 through January 2006. A family history of allergic diseases was defined according to the following criteria: (i) when at least one parent or older sibling of the fetus had eczema, as confirmed by a pediatric allergist at enrollment; or (ii) when one of the parents had been diagnosed with asthma and/or allergic rhinitis by a physician, showing house dust mite-specific IgE over 1.0 kU/L by CAP-FEIA immunoassay (Pharmacia, Uppsala, Sweden).

Treatment of either probiotics or placebo was allocated by trials coordinator without detailed knowledge of the clinical history according to computerized randomization. The groups were stratified and block-randomized in accordance with (1) maternal allergy (allergy vs. no allergy), (2) older sibling's eczema, and (3) number of parents affected by allergic disease (1 vs. 2) (Table 1).

Mothers in the probiotics group took a mixture of *Bifidobacterium bifidum* BGN4 [1.6×10^9 colony forming units (CFU)], *Bifidobacterium lactis* AD011 (1.6×10^9 CFU), and *Lactobacillus acidophilus* AD031 (1.6×10^9 CFU) in 0.72 g of maltodextrin and 0.8 g of alpha-corn (Bifido Inc., Hongchungun, Korea) once daily from 8 wks before the expected delivery to 3 months after delivery. Infants were fed the same powder dissolved in breast milk, infant formula, or sterile water from 4 to 6 months of age. Mothers and infants in the placebo group took maltodextrin and alpha-corn without probiotic bacteria. The

Table 1. Baseline characteristics of the participants at the time of randomization

	Probiotics group n (%)	Placebo group n (%)
Enrolled number	57	55
Mother's age (yrs)*	29.93 ± 0.37	29.53 ± 0.45
Maternal allergic diseases	43 (75.4)	45 (81.8)
Parental history of allergic disease		
Biparents	22 (38.6)	14 (25.5)
Single parent	33 (57.9)	38 (69.1)
Allergic diseases in participant's family		
Eczema	34 (59.6)	38 (69.1)
AR or asthma	39 (68.4)	27 (49.1)
House dust mite-specific IgE of parents (kU/L)*		
D. pteronyssinus	6.63 ± 1.37	9.60 ± 2.56
D. farinae	10.41 ± 2.52	12.71 ± 3.01
Paternal smoking	14 (24.6)	19 (34.5)

*Mean ± s.e.m.

There were no significant differences between the groups for any of the variables determined by Student's *t* test for continuous data and Pearson's chi-square test for all nominal data.

AR, allergic rhinitis.

probiotic and placebo sachets and contents looked, smelled, and tasted identical. Compliance was monitored by recording the date at which the administration period was discontinued, and counting the remaining sachets. All mothers were requested to breastfeed their infants for at least 3 months after birth; Thereafter, they were permitted to feed their infants with cow's milk formula. Lactating mothers and infants were prevented from eating peanuts and eggs, as well as yogurt and other probiotic functional foods, during the course of the study.

Subjects were excluded if they met any one of the following exclusion criteria: (1) premature babies delivered at less than 36 wks of gestation; (2) infants with immune deficiency disease, necrotizing enterocolitis, or congenital disorders; or (3) those requiring anticancer treatment or a central venous catheter. In the current study, one infant with a congenital disorder was excluded.

The study protocol was approved by the Ethical Committee at the Samsung Medical Center. Written informed consent was obtained from all participants. Our study protocol was registered in ISRCTN (International Standard Randomised Controlled Trial Number). The registration number is ISRCTN26134979.

Clinical assessments

Infants were followed up to 1 yr of age, involving examinations at 3, 6, and 12 months to assess the occurrence of eczema, the main atopic disease during infancy. Diagnosis of eczema was confirmed when the skin lesions met the criteria of Hanifin and Rajka (16). The severity of eczema was determined using the Six Area Six Sign in Atopic dermatitis (SASSAD) score (17). We also collected data on various clinical histories such as gestational age, birth weight, cesarean section delivery, mother's age, duration of breastfeeding, diet, fever (≥38.5°C), respiratory tract infection, hospitalization, acute gastroenteritis, and use of antibiotics. Diagnosis and clinical assessment of eczema was performed by a pediatric allergist who remained unaware of the actual treatment administered during the entire study period.

Venous blood was obtained at 12 months of age to measure total IgE and specific IgE against common food allergens (egg white, cow's milk, wheat, peanut, soybean, and buckwheat) using CAP-FEIA (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Antigen-specific IgE levels greater than 0.35 kU/L were considered positive. 'Probable egg allergy' was defined when the egg white-specific IgE level was 2 kU/L or higher based on the 95% positive predictive value (18). Likewise, infants were diagnosed as having 'probable cow's milk allergy' when they showed cow's milk-specific IgE of 5 kU/L or higher.

Statistical analysis

We anticipated that this high-risk population would have a 60% cumulative incidence of eczema at an early age, and that intervention with probiotics could reduce the proportion of cases developing allergic diseases to 30%. The study design had more than 80% power at the 5% significance level; the estimated size of each group was 55, which allowed for a 15% dropout rate.

Pearson's chi-square test was used to compare the prevalence of outcome variables and background factors between the two groups. Logistic regression analysis was performed to compare the prevalence of eczema and food allergy, and multivariable logistic regression was used to adjust for potential confounding factors (cesarean delivery, breastfeeding, use of antibiotics). The results are given as Odds Ratio (OR) with a 95% Confidence Interval (CI). Total IgE and allergen-specific IgE of parents were normally distributed and assessed using Student's *t*-test, expressed as mean and standard error of the mean (s.e.m.). All statistical analyses were performed using SPSS 12.0K for Windows (SPSS Inc., Chicago, IL, USA). A p value of < 0.05 was considered to be statistically significant.

Results

Characteristics of the participants

A flow chart to show the progress of the present study is displayed in Fig. 1. Among the 159 pregnant women who took a blood test and filled in the questionnaire, 112 who met the inclusion criteria were randomized and divided into the probiotics (n = 57) and placebo (n = 55) groups. Prenatally, no mother discontinued administration of the sachets; overall, 90% of the sachets were administered in both groups. During the postnatal period, two infants in the probiotics group and one in the placebo group were excluded because of poor compliance (less than 70%); approximately 85% of the sachets were administered, with no difference in compliance between the groups. The actually treated prenatal periods were 53.62 ± 1.75 days in probiotics group and 52.29 ± 1.33 days in placebo group. The actual postnatal period of treatment was 180.94 ± 5.21 days in probiotics group and

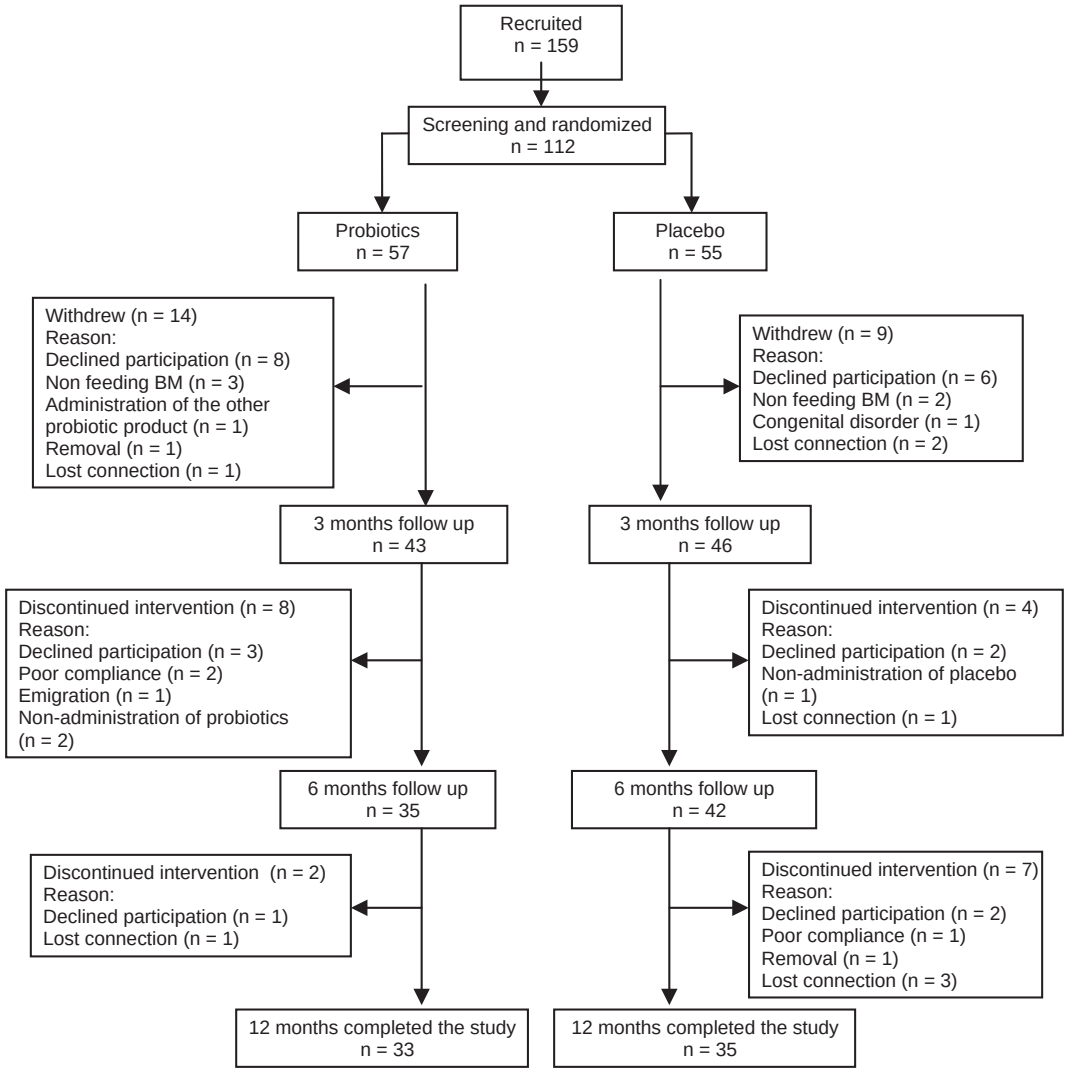

Fig. 1. Flow chart showing the progress of the study.

181.03 ± 5.06 days in placebo group. A total of 44 participants (39.3%) discontinued and ultimately, 68 mothers and their babies (33 in the probiotics group, 35 in the placebo group) completed the study (i.e., participated until the baby was 12 months of age).

There was no difference at the time of randomization between the two groups with respect to mother's age, maternal allergic diseases, biparental allergic diseases, family history of eczema, paternal smoking, or house dust mite-specific IgE levels of parents (Table 1). There was also no significant difference in terms of gestational age, birth weight, gender, presence of older siblings, infections, antibiotic use, and hospitalization during infancy between the two groups when only those participants successfully followed to 12 months were included (Table 2). The rate of cesarean delivery in the probiotics group was half that in the placebo group (15.2% vs. 31.4%; $p = 0.114$), and the total duration of the breastfeeding in the probiotics group was longer than in the placebo group (9.41 ± 0.61 vs. 7.69 ± 0.70 months; $p = 0.068$). Although these findings were not statistically significant, they were considered as potential confounding factors in subsequent analyses.

In the current study, the parents were asked to report any adverse effects whenever they happen. No serious adverse effects developed and although non-specific mild symptoms developed, these were unlikely to have been related to the administration of probiotics.

Effects of probiotics on development of eczema

The prevalence of eczema was not significantly different between the two groups at 3 months of

Table 2. Base and clinical characterization of infants who completed the 1 yr-study

	Probiotics group n (%)	Placebo group n (%)
Number at the end of study	33	35
Gestational age (wks)*	39.66 ± 0.25	39.47 ± 0.19
Birth weight (kg)*	3.33 ± 0.07	3.25 ± 0.06
Gender (boys/girls)	15/18	15/20
Cesarean section delivery	5 (15.2)	11 (31.4)
Duration of breast-feeding (month)*		
Exclusive breast-feeding	5.97 ± 0.89	5.26 ± 0.88
Total breast-feeding	9.41 ± 0.61	7.69 ± 0.70
Presence of older sibling	4 (12.1)	6 (17.1)
Infections during follow-up		
Fever (≥38.5°C)	13 (39.4)	11 (31.4)
Respiratory tract infection	26 (78.8)	26 (74.3)
Acute gastroenteritis	11 (33.3)	7 (20.0)
Antibiotics use during follow-up	13 (39.4)	11 (31.4)
Hospitalization during follow-up	6 (18.2)	6 (17.1)

*Mean ± s.e.m.
There were no significant differences between the groups for any of the variables determined by Student's *t* test for continuous data and Pearson's chi-square test for all nominal data.

age, up to which point lactating mothers were given the probiotics or placebo (probiotics group, 18.6% vs. placebo group, 34.8%; p = 0.086). At 6 months of age, when the infants were receiving formula supplemented with probiotics or placebo, the prevalence rate of eczema in the probiotics group was half that in the placebo group (20.0% vs. 40.5%; p = 0.053). At 12 months of age, the prevalence rate of eczema in the probiotics group was reduced to less than half that in the placebo group; this result was statistically significant (18.2% vs. 40.0%; p = 0.048; Table 3).The cumulative incidence of eczema in the probiotics group was significantly lower than that in the placebo group at 12 months of age (36.4% vs. 62.9%; p = 0.029; Table 3). This result demonstrated that probiotics protected the infants from developing eczema.

The severity of skin lesions was assessed only in those with eczema by SASSAD, and compared between the two groups. Those without eczema were not scored. There was no significant difference in the severity of eczema between the two groups.

IgE sensitization

Sera were obtained from 31 infants in the probiotics group and 29 in the placebo group to measure total and specific IgE; eight mothers (two from the probiotics group, six from the placebo group) refused to withdraw blood from their infants. Total IgE level and the frequency of sensitization against common food allergens was similar in both groups, suggesting that probiotics did not affect sensitization (Table 4). The prevalence rate of IgE-associated eczema or atopic eczema in the probiotics group was half that in the placebo group, although this was not significantly different (9.7% vs. 20.7%, p = 0.233). 'Probable egg allergy' or 'probable cow's milk allergy' occurred similarly in both groups (Table 4).

Discussion

In this double-blind, randomized, placebo-controlled study, we demonstrated the preventive effect of mixed probiotics (*B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031) on development of eczema in infants at high risk of atopic diseases. *B. bifidum, B. lactis*, and *L. acidophilus* have been detected in samples from healthy humans (19, 20), and these probiotic bacteria were used in our study. *B. bifidum* BGN4 exhibited a prominent adhesive capacity for intestinal epithelial cells, which is one of the desirable properties for a probiotic effect (21). In the CD4 + $CD45RB^{high}$ T cell transfer inflammatory bowel disease model, *B. bifidum*

Table 3. Cross-sectional prevalence and cumulative incidence of eczema at 3, 6, and 12 months of age

	Probiotics group	Placebo group	p-value	Adjusted OR(95% CI)	p-value
Cross-sectional prevalence					
3 months	8/43 (18.6%)	16/46 (34.8%)	0.086	0.511 (0.178–1.468)	0.212
6 months	7/35 (20.0%)	17/42 (40.5%)	0.053	0.377(0.119–1.197)	0.098
12 months	6/33 (18.2%)	14/35 (40.0%)	0.048*	0.256(0.067–0.976)	0.046†
Cumulative incidence at 12 months	12/33 (36.4%)	22/35 (62.9%)	0.029*	0.243(0.075–0.792)	0.019†

*Significant difference between the groups as determined by Pearson's chi-square test.
†p value was calculated by multivariable logistic regression analysis adjusted for the antibiotics use, total duration of breastfeeding, and delivery by cesarean section.

Table 4. Comparison of sensitization and prevalence of probable food allergy between two groups

	Probiotics group n (%)	Placebo group n (%)	p-value
Number who completed blood test	31	29	
Total Serum IgE (kU/L)*	111.7 ± 58.9	80.6 ± 28.7	0.638
Food-specific IgE (≥0.35 kU/L)			
Any food	12 (38.7)	15 (51.7)	0.311
Egg white	9 (29.0)	8 (27.6)	0.901
Cow's milk	9 (29.0)	11 (37.9)	0.465
Soybean	3 (9.7)	3 (10.3)	0.931
Wheat	2 (6.5)	2 (6.9)	0.946
Peanut	1 (3.2)	4 (13.8)	0.139
Buckwheat	1 (3.2)	2 (6.9)	0.514
IgE-associated eczema	3 (9.7)	6 (20.7)	0.233
Probable food allergy			
Egg	4 (12.9)	4 (13.8)	0.919
Cow's milk	1 (3.2)	0 (0.0)	0.329

*Mean ± s.e.m.
There were no significant differences between the groups for any of the variables determined by Student's *t* test for continuous data and Pearson's chi-square test for all nominal data.

BGN4-fed mice showed suppression of inflammatory cell infiltration and reduced levels of CD4 + T lymphocyte infiltration and inflammatory cytokine production compared with skim milk-fed mice (22). Orally administered *B. bifidum* BGN4 also prevented IgE-mediated ovalbumin-induced allergy in C3H/HeJ mice (23). These findings suggest that *B. bifidum* BGN4 supplementation could be helpful in the control of aberrant immune responses.

In our preliminary study, mice fed with *B. lactis* AD011 and *L. acidophilus* AD031 showed suppressed levels of ovalbumin-specific IgE in serum, reduced concentrations of IL-4 and increased concentrations of IL-10 and IFN-γ in spleen cell culture assay on ovalbumin-induced allergy model (24). Additionally, *B. lactis* AD011 and *L. acidophilus* AD031 increased the production of IL-10 in dendritic cells (not published). Previous studies also have reported that the administration of *B. lactis* or *L. acidophilus* species alleviated the symptoms of allergic diseases. For example, supplementation with *B. lactis* Bb-12 reduced the severity of atopic eczema in infants (25), and oral administration of fermented milk containing *L. acidophilus* L-92 improved allergic rhinitis (26). Based on these results, we attempted to prevent the development of eczema with mixture of beneficial probiotics (*B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031), and found these probiotics could be used for the infants at high risk of developing eczema.

Recent study revealed that supplementation with *L. acidophilus* (LAVRI-A1) did not prevent atopic dermatitis, but instead led to an increased frequency of common antigen sensitization in infants at high risk of allergy by 1 yr of age (15). This contradictory result may be attributed to different study population, the use of different strain or no prenatal administration of probiotics, despite using the same species, *L. acidophilus*. However, further studies are necessary to verify which species or strains are most beneficial, because our study used mixture of those probiotics, not a single strain.

Several clinical trials have investigated whether prenatal or indirect supplementation with probiotics via breastfeeding could enhance the primary prevention of eczema at an early age. When prenatal and breastfeeding mothers were supplemented with *L. rhamnosus* GG from 2–4 wks before delivery, the rate of their infants' atopic eczema at 2 yrs of age was half that of those supplemented with the placebo (13). Infants born from mothers supplemented with *L. reuteri* for 4 wks before delivery showed a lower frequency of IgE-associated eczema and positive reaction to a skin prick test than the placebo group (27). The above results may well suggest that maternal immunocompetence has an effect *in utero* or on breastfeeding infants. Recent study showed that maternal probiotic supplementation reduced sensitization in infants at high risk of developing allergic diseases and that might have been related to the change in the composition of breast milk such as TGF-β2 (28).

In this prospective study, the frequency of positive food antigen-specific IgE sensitization and food allergy in Korean infants at high risk was not reduced by supplementation with probiotics. This result was consistent with that of the previous study (29), although the reason for the discrepancy between development of eczema and sensitization against food allergens was not clear. However, our data showed that sensitization against any one of common food allergen appeared to be lower in probiotics group (38.7%) than in placebo group (51.7%). The prevalence of IgE-associated eczema also seemed lower in probiotics group (9.7% vs. 20.7%). Still it might be possible to demonstrate the effect of probiotics on the prevention of sensitization if investigations with larger study population are conducted.

Our study is limited by the high drop-out rate. Some participants stopped participating in the study without explanation, or moved from the area. In addition, we excluded all participants who did not adhere to our protocol. Consequently, a high proportion of participants failed

to complete the study. Intention-to-treat analysis may be helpful in such a situation; however, the full application of intention-to-treat analysis is possible only when complete outcome data are available for all randomized subjects. In the present study, however, data are missing regarding the primary outcome for discontinued participants, and the methods employed to deal with this problem were inadequate, potentially leading to bias. We analyzed a total of 44 participants who discontinued or withdrew from the study. Twenty-three participants dropped out before the first follow-up, and their development of eczema was not identified. Among 21 infants who discontinued the study after 3 months of age, the occurrence of eczema was 20.0% (2/10) in the probiotics group and 54.5% (6/11) in the placebo group at their last visit. Considering that the prevalence or cumulative incidence of eczema in the probiotics group is less than half that in the placebo group, the administration of probiotics in the discontinued participants appears to have had a similar preventive effect to that in those who completed the study. In addition, among those who successfully completed the 1-yr study, there was no difference in baseline characteristics or drop-out rate between the probiotics group and placebo group. Therefore, our results suggest that a probiotic mixture was beneficial in preventing eczema, despite the high drop-out rate.

This study showed high prevalence of eczema. The prevalence (40.0%) at 1 yr and cumulative incidence (62.9%) of eczema in placebo group seemed high in the present study. Other studies also showed that the frequency of eczema in placebo group was as high as 45–46% (10, 13). We obtained similar data in our previous study where the prevalence of eczema was 59% and 41% in cow's milk formula-fed group and breastmilk-fed group, respectively, in infants at high risk of eczema (30). Probably high incidence of eczema might have been due to the selection of infants with high risk of developing atopic disease.

Additionally, our study ended when the infants were 1 yr of age. Although we could find lower incidence of eczema in probiotics group, it is not clear whether this preventive effect persists as they grow older.

In conclusion, mixture of probiotics (*B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031) have beneficial effect to prevent development of eczema in infants at high risk during their first year of life. Further studies are needed to understand the basic mechanisms of probiotics in the primary prevention of eczema.

Acknowledgments

This work was supported by grants (A060546 and A080664) from the Ministry for Health, Welfare and Family Affairs, Republic of Korea.

References

1. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. Lancet 1998: 351: 1225–32.
2. Asher MI, Montefort S, Bjorksten B, et al. ISAAC Phase Three Study Group. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006: 26: 733–43.
3. Oh JW, Pyun BY, Chong JT, et al. Epidemioligical change of atopic dermatitis and food allergy in school-aged children in Korea between 1995 and 2000. J Korean Med Sci 2004: 19: 716–23.
4. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. New Engl J Med 2002: 347: 911–20.
5. Bufford JD, Gern JE. The hygiene hypothesis revisited. Immunol Allergy Clin North Am 2005: 25: 247–62.
6. Rautava S, Ruuskanen O, Ouwehand A, Salminen S, Isolauri E. The hygiene hypothesis of atopic disease-An extended version. J Pediatr Gastroenterol Nutr 2004: 38: 378–88.
7. Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol 1997: 159: 1739–45.
8. Bjorksten B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy 1999: 29: 342–6.
9. Kalliomaki M, Kirjavainen P, Eerola E, Kero P, Salmonen S, Isolauri E. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J Allergy Clin Immunol 2001: 107: 129–34.
10. Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol 2001: 108: 516–20.
11. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to Toll-like Receptor bacterial ligands in intestinal epithelial cells. Gastroenterology 2004: 126: 1054–70.
12. Rook GAW, Brunet LR. Microbes, immunoregulation, and the gut. Gut 2005: 54: 317–20.
13. Kalliomaki M, Salminen S, Arvilommi H, Kero P, koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 2001: 357: 1076–9.
14. Kalliomaki M, Salminen S, Poussa T, Isolauri E. Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. J Allergy Clin Immunol 2007: 119: 1019–21.
15. Taylor AL, Dunstan JA, Prescott SL. Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the

risk of allergen sensitization in high-risk children: a randomized controlled trial. J Allergy Clin Immunol 2007: 119: 184–91.
16. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venerol (stockh) 1980: 92: 44–7.
17. Berth-Jones J. Six area, six sign atopic dermatitis (SASSAD) severity score: a simple system for monitoring disease activity in atopic dermatitis. Br J Dermatol 1996: 48: 25–30.
18. Sampson HA. Update on food allergy. J Allergy Clin Immunol 2004: 113: 805–19.
19. Gilliland SE, Morellil, Reid D. Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk With Live Lactic Acid Bacteria. Cordoba, Argentina: WHO, 2001.
20. Savino F, Bailo E, Oggero R, et al. Bacterial counts of intestinal Lactobacillus species in infants with colic. Pediatr Allergy Immunol 2005: 16: 72–5.
21. Kim IH, Park MS, Ji GE. Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 Cells. J Micobiol Biotechnol 2003: 13: 276–81.
22. Kim N, Kunisawa J, Kweon MN, Ji GE, Kiyono H. Oral feeding of *Bifidobacterium bifidum* (BGN4) prevents CD4 + CD45RBhigh T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. Clin Immunol 2007: 123: 30–9.
23. Kim H, Kwack K, Kim DY, Ji GE. Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model. FEMS Immunol Med Microbiol 2005: 45: 259–67.
24. Kim JY, Choi YO, Ji GE. Effect of oral probiotics (*Bifidobacterium lactis* AD011 and *Lactobacillus acidophilus* AD031) administration on ovalbumin-induced food allergy mouse model. J Microbiol Biotechnol 2008: 18: 1393–400.
25. Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. Clin Exp Allergy 2000: 30: 1604–10.
26. Ishida Y, Nakamura F, Kanzato H, et al. Clinical effects of *Lactobacillus acidophilus* strain L-92 on perennial allergic rhinitis: a double-blind, placebo-controlled study. J Dairy Sci 2005: 88: 527–33.
27. Abrahamsson TR, Jakobsson T, Bottcher MF, et al. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol 2007: 119: 1174–80.
28. Bottcher MF, Abrahamsson TR, Fredriksson M, Jakobsson T, Bjorksten B. Low breast milk TGF-b2 is induced by *Lactobacillus reuteri* supplementation and associates with reduced risk of sensitization during infancy. Pediatr Allergy Immunol 2008: 19: 497–504.
29. Rautava S, Kalliomaki M, Isolauri E. Probiotics during pregnancy and breast-feeding might confer immunomudulatory protection against atopic disease in the infant. J Allergy Clin Immunol 2002: 109: 119–21.
30. Han YS, Park HY, Ahn KM, Lee JS, Choi HM, Lee SI. Short-term effect of partially hydrolyzed formula on the prevention of development of atopic dermatitis in infants at high risk. J Korean Med Sci 2003: 18: 547–51.

ORIGINAL ARTICLE

Gut and Liver, Vol. 3, No. 2, June 2009, pp. 101-107

Effect of Probiotics on Symptoms in Korean Adults with Irritable Bowel Syndrome

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Background/Aims: Irritable bowel syndrome (IBS) is a troublesome disease. Some strains of probiotics reportedly exert remarkable immunomodulatory effects, and so we designed a prospective double-blind randomized placebo-controlled clinical study to assess their effects in Korean adults with IBS. **Methods:** IBS patients who met Rome III criteria were randomly assigned to receive composite probiotics or placebo. A total of 20 billion lyophilized bacteria were administered twice daily for 8 weeks. Primary outcome variables were symptom scores consisting of abdominal pain, flatulence, defecation discomfort, and sum of symptom scores. A visual analogue scale was used to quantify the severity. Secondary outcome variables consisted of the quality of life and bowel habits including defecation frequency and stool form. **Results:** Thirty-six and 34 patients were randomized to the probiotics and placebo groups, respectively. Intention-to-treat analysis showed significant reductions in pain after 8 weeks of treatment: −31.9 and −17.7 in the probiotics and placebo groups, respectively (p=0.045). The reductions in abdominal pain, defecation discomfort, and sum of scores were more significant in 58 patients with a score of at least 3 on the baseline stool-form scale. **Conclusions:** Composite probiotics containing *Bifidobacterium bifidum* BGN4, *Lactobacillus acidophilus* AD031, and other species are safe and effective, especially in patients who excrete normal or loose stools. **(Gut and Liver 2009;3:101-107)**

Key Words: Probiotics; Irritable bowel syndrome; *Bifidobacterium bifidum*

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most troublesome diseases, which has the high prevalence as well as the chronic and recurrent course. In United States, IBS is known as the most common gastrointestinal disease and comprises 25 to 50% of all referrals to gastroenterologists,[1] and the prevalence of IBS has estimated to range 9% to 22% of the population.[2] In Korea, Park *et al.*[3] reported the prevalence of IBS increased up to 16.8%. A few effective medicines such as cisapride, tegaserod and alosetron have been withdrawn from the market because of their serious adverse drug reactions. There is no specific treatment that has proven to be effective and safe in the patients with IBS.

Through previous studies, IBS is known to be associated with low-grade inflammation of the intestinal mucosa regardless of whether to be the post-infectious subtype or not.[4,5] Although a few clinical studies to evaluate the immunomodulatory effect of probiotics showed symptom relief in IBS patients, probiotics are not yet used widely in daily practice.[6-8] As global competetion for searching more potent strain was heating up, a well-designed clinical study becomed necessary to validate the effect of promising probiotics. Two *in vitro* studies using some strains of *Bifidobacterium bifidum* demonstrated that they were effective in inhibiting lipopolysaccharide (LPS)-induced inflammation, and the later study showed high LPS-binding capacity and inhibition of inflammatory cytokine.[9,10] Kim *et al.*[11] reported *Bifidobacterium bifidum* BGN4 strain had the significant immunomodulatory effect

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Received on February 23, 2009. Accepted on April 10, 2009.

on the control of inflammatory bowel disease (IBD) using a mouse model. With composite probiotics including *Bifidobacterium bifidum* BGN4 and other promising strains, we designed a prospective double-blind randomized placebo-controlled clinical study to prove the effect in the Korean adults with IBS.

MATERIALS AND METHODS

1. Study population

Patients were recruited from the outpatient department of Seoul National University Hospital. The study protocol was approved by the institutional review board. The inclusion criteria were as follows: age of 19-75 years, both male and female, and the presence of previous gastrointestinal symptoms suggestive of IBS using the Rome III criteria regardless of its subtypes. All participants gave a written informed consent form that had been approved by the institutional review board.

The exclusion criteria were as follows: previous abdominal surgery except appendectomy and hernia repair, history of IBD, current use of medications that may alter gastrointestinal motility, antibiotics or probiotics within 2 weeks prior to the 1-week run-in period, severe co-morbidity such as cancer, heart or renal failure, gynecologic disease etc., and pregnant or breast-feeding female.

2. Study design

We performed a parallel-group, double-blinded, randomized, placebo-controlled clinical study. A 1-week run-in observation period was followed by an 8-week treatment period. During this entire 9-week period, participants were required to record a daily diary of bowel habits consisting of frequency and consistency. A questionnaire on irritable bowel symptoms such as abdominal pain, flatulence and defecation discomfort was recorded at baseline, 4th and 8th week after treatment. A questionnaire on quality-of-life (QOL) was recorded at baseline and 8th week.

Primary outcome variables were symptom scores that consisted of abdominal pain, flatulence, defecation discomfort and the sum of these three symptom scores. Laborious evacuation, tenesmus and urgency were included in questionnaire for defecation discomfort. A 100 mm visual analogue scale (VAS) was used to measure the severity of each symptom as scores ranging from 0 to 100. When participants were asked to mark VAS after treatment, they could look at his or her previous marks. Secondary outcome variables were bowel habits that recorded using a validated Bristol stool form scale and QOL that recorded using a RAND 36-item health survey.[12-14]

3. Administration of probiotics

Composite probiotics were composed of 4 viable lyophilized bacteria species: *Bifidobacterium bifidum* BGN4; *Bifidobacterium lactis* AD011; *Lactobacillus acidophilus* AD031; and *Lactobacillus casei* IBS041. Each probiotic packet with equal doses of 4 strains contained total 20 billion lyophilized bacteria in a powder form. A placebo packet containing skim milk powder looked identical to the composite probiotics. Both probiotics and placebo were supplied by BIFIDO Co., Ltd., Hongchun, Korea. Each participant in both treatment groups received one packet orally with water within 10 minutes after a meal, twice daily (40 billion lyophilized bacteria per day) for 8 weeks.

4. Randomization

We used blocked randomization method with block size 4 or 6 and generated permutations at random using SPSS for Windows 12.0.1 (SPSS Inc., Chicago, IL, USA) provided by the medical research collaborating center at our institution that was independent of medical care. All participants were assigned an allocation number in regular sequence after confirmation of enrollment. For adherence to double-blind design, the allocation number was matched to a randomization code successively by a clinical trial pharmacist. Till completion of the study, the clinical trial pharmacist kept the randomization table sealed off.

5. Sample size calculation and statistical analyses

On the ground of previous reports, we assumed the response rates would be 70% in probiotics group and 40% in placebo group.[8] The response was defined as reduction of symptom score by at least 50% after treatment. Other assumptions for sample size calculation were as follows: alpha error 0.05; statistical power 0.8; drop-out rate 0.05; and one-sided test. We used the equation below, the sample size was estimated as 35 per group in view of drop-out rate.

$$N = \frac{\left(Z_\alpha \sqrt{2p(1-p)} + Z_\beta \sqrt{p_1(1-p_1) + p_2(1-p_2)}\right)^2}{d^2}$$

p_1 (response rate in probiotics group)=0.7
p_2 (response rate in placebo group)=0.4
$p=(p_1+p_2)/2=0.55$
$d=p_2-p_1=0.3$
$Z_\alpha=1.65$ (alpha error=0.05)
$Z_\beta=0.84$ (statistical power=0.8)

All data were collected by a single trained interviewer who was a clinical research coordinator. Week 0 (the end

of run-in phase) was considered as baseline in all statistical analyses. The "intent-to-treat" (ITT) population was defined as all participants who received probiotics or placebo for at least one week and visited our hospital for the interview once or more. Efficacy analysis was performed in the ITT population. χ^2-test was performed to test response rates. As symptom score, QOL score, consistency and frequency were all continuous variables, two-sided T-test was performed using the 0.05 significance level.

RESULTS

Between 1 November 2007 and 29 February 2008, 76 patients were screened. 5 patients (6.5%) were ineligible as they did not meet the inclusion criteria, and 1 withdrew consent. A total of 70 patients were enrolled, 36 were randomized to probiotics group and 34 to placebo. Demographic and clinical characteristics were similar between the two groups (Table 1).

1. Compliance and concomitant medications

Of 36 participants assigned to probiotics, 35 completed treatment as planned. One participant withdrew from the study due to an exacerbation of abdominal pain associated with IBS. Another one participant, who was assigned to placebo, withdrew from the study due to an exacerbation of constipation associated with IBS. Both withdrawn participants were included in efficacy analysis based on the definition of ITT population; symptom scores and the bowel habit were imputed using the mean value of the group. A total of 68 participants completed the study. Overall compliance was more than 98% in both groups.

Eleven of 70 participants required concomitant medications during the study, 3 in probiotics group and 8 in placebo. Ten participants, except one in probiotics group who was prescribed a common cold medication, used loperamide, prokinetics, pain killers, histamine 2 receptor antagonists, proton pump inhibitors or laxatives due to an exacerbation of bowel symptoms. According to ITT principle, all the eleven patients were included in analyses.

2. Symptom scores, QOL and bowel habits

ITT analyses showed significant reductions of pain score after 8 weeks of treatment (−31.9 in probiotics group vs. −17.7 in placebo [p=0.045]) and defecation discomfort after 4 weeks of treatment (−29.2 vs. −13.5, respectively [p=0.043]). Subgroup analyses in 58 patients whose baseline Bristol stool form scales were 3 or more

Table 1. Characteristics of Subjects

Characteristics	Probiotics (n=36)	Placebo (n=34)
Age (years)		
Mean (±SE)	36±2	38±3
Range	21-69	22-72
Females (n)	11	12
Baseline score (±SE)		
Pain	50.3 (±3.5)	46.9 (±3.4)
Flatulence	49.9 (±3.6)	49.3 (±4.8)
Defecation	53.5 (±4.0)	47.0 (±5.1)
Sum	153.6 (±8.9)	143.2 (±9.0)
QOL	104.6 (±1.1)	104.7 (±1.4)
Defecation frequency*	8.1	7.1
Stool consistency†	4.2	4.0
IBS subtype‡		
Diarrhea	19	13
Constipation	7	7
Mixed	2	4
Unsubtyped	8	10

QOL, quality-of-life.
*Bowel movements per week, †Bristol stool form scale; average for a week, ‡Rome III criteria.

Table 2. Symptom Scores Analyzed over 4 and 8-week Treatment Period (Two-sided T-test Using the 0.05 Significance Level)

		Probiotics	Placebo	p-value
ITT population (n=70)				
Pain	Baseline	50.3	46.9	0.487
	⊿ 4 week	−23.9	−10.9	0.061
	⊿ 8 week	−31.9	−17.7	0.045
Flatulence	Baseline	49.9	49.3	0.928
	⊿ 4 week	−18.5	−18.4	0.982
	⊿ 8 week	−27.0	−21.3	0.437
Defecation	Baseline	53.5	47.0	0.311
	⊿ 4 week	−29.2	−13.5	0.043
	⊿ 8 week	−30.5	−18.4	0.122
Sum	Baseline	153.6	143.2	0.413
	⊿ 4 week	−71.7	−42.8	0.115
	⊿ 8 week	−89.5	−57.5	0.064
Subgroup* (n=58)				
Pain	Baseline	51.2	46.2	0.315
	⊿ 4 week	−26.9	−5.8	0.004
	⊿ 8 week	−33.9	−13.3	0.006
Flatulence	Baseline	49.2	47.8	0.826
	⊿ 4 week	−19.6	−13.0	0.463
	⊿ 8 week	−26.7	−15.9	0.175
Defecation	Baseline	53.2	45.0	0.826
	⊿ 4 week	−30.4	−10.6	0.013
	⊿ 8 week	−30.0	−14.5	0.064
Sum	Baseline	153.6	138.9	0.288
	⊿ 4 week	−76.9	−29.4	0.010
	⊿ 8 week	−90.6	−43.6	0.010

*Baseline Bristol stool form scale ≥3.

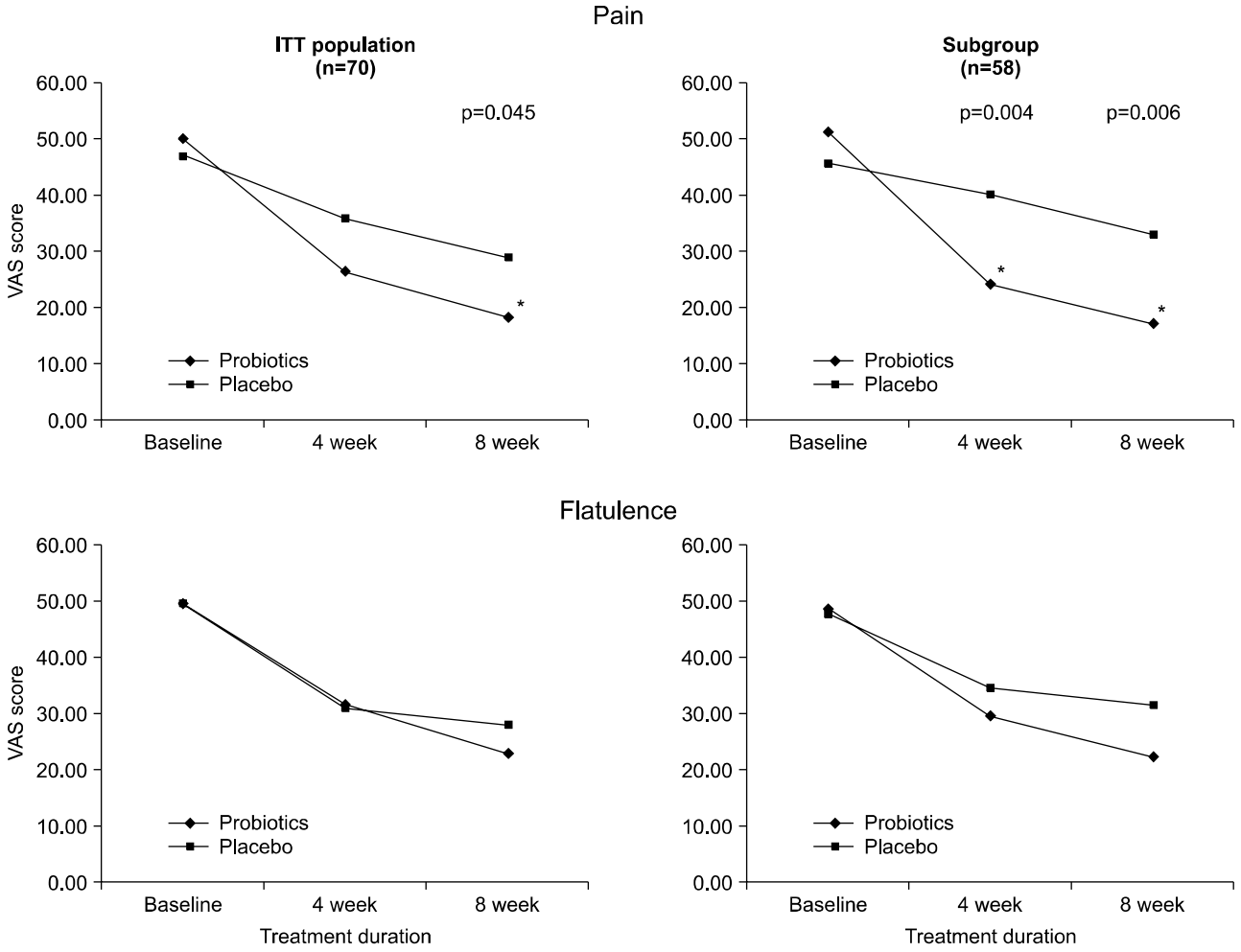

Fig. 1. Pain and flatulence scores analyzed over 4- and 8-week treatment periods.

showed more significant reductions of pain score after 8 weeks of treatment (−33.9 in probiotics group vs. −13.3 in placebo [p=0.006]), defecation discomfort score after 4 weeks of treatment (−30.4 vs. −10.6, respectively [p=0.013]), and sum of scores after 8 weeks of treatment (−90.6 vs. −43.6, respectively [p=0.010]) (Table 2, Figs. 1 and 2). Subgroup analyses in 10 patients, whose baseline Bristol stool form scales were below 3, did not show any significant changes. Response rate evaluation through χ^2-test failed to show significant changes as follows: response rate in pain were 64% in probiotics group vs. 44% in placebo (p=0.248), response rate in defecation discomfort were 58% vs. 41% (p=0.317), and response rate in sum of scores were 56% vs. 50% (p=0.750), respectively. There was no significant change of QOL and bowel habits including defecation frequency and stool consistency in both groups (Table 3).

3. Adverse events

There was no serious adverse event associated with treatments. Twelve of 70 participants reported mild adverse events including common cold, headache, cystitis, low back pain etc. The number of adverse events per group was 8, same in both groups.

DISCUSSION

Symptoms of IBS are subjective and there is no objective test that can measure severity of IBS. Symptom scores are popular methods for assessing severity of IBS, but these can be influenced by an interviewer as well as patients themselves. Strict double-blind design is essential to assess the effect of probiotics on symptoms in IBS patients. We could perform a strict double-blinded study by the help of MRCC and clinical trial pharmacists for random code generation and code-matching. Patel *et al.*[15]

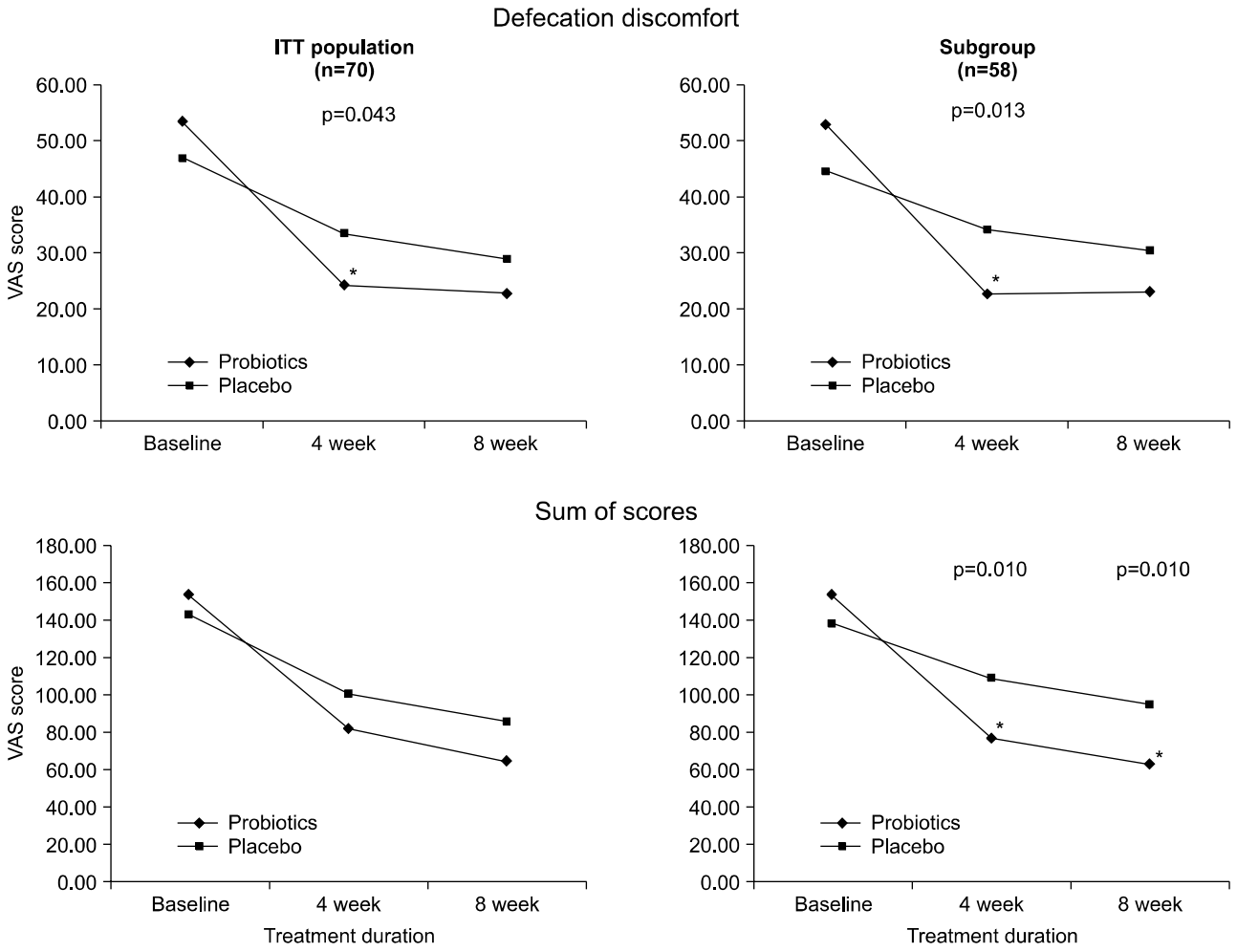

Fig. 2. Defecation discomfort and sum of scores analyzed over 4- and 8-week treatment periods.

Table 3. Analysis of Quality of Life, Defecation Frequency and Stool Consistency over 8-week Treatment Period (Two-sided T-test Using the 0.05 Significance Level)

		Probiotics	Placebo	p-value
ITT population (n=70)				
QOL	Baseline	104.6	104.7	0.957
	⊿ 8 week	−1.3	−1.1	0.887
Frequency†	Baseline	8.1	7.1	0.414
	⊿ 8 week	−0.1	0.1	0.817
Consistency‡	Baseline	4.2	4.0	0.453
	⊿ 8 week	0.2	−0.1	0.430
Subgroup* (n=58)				
QOL	Baseline	104.4	104.8	0.876
	⊿ 8 week	−1.5	−1.7	0.919
Frequency†	Baseline	8.7	7.9	0.553
	⊿ 8 week	−0.6	−0.2	0.689
Consistency‡	Baseline	4.6	4.4	0.374
	⊿ 8 week	−0.1	−0.4	0.241

QOL, quality-of-life.
*Baseline Bristol stool form scale ≥3, †Bowel movements per week, ‡Bristol stool form scale; average for a week.

reported that placebo response in IBS studies ranged from 16% to 71% via meta-analysis. In our study, the overall placebo effect was 35% that is comparable with many other IBS studies and seems to be a matter of course in double-blinded study.[16-18] Strong points of our study design include strict double-blind design, data collection by a single trained interviewer, relatively large number of study population (n=70) and no need of data processing including adjusting and standardization.

Although Kim *et al.*[6,7] reported that VSL #3 reduced flatulence scores in the patients with IBS, probiotics have not been the standard treatment of IBS due to the following reasons: need of huge dose of probiotics (VSL #3, 4.5×10^{11} bacteria/packet), relatively low efficacy (10 mm difference from placebo on 100 mm scale) and no effect in abdominal pain and urgency. A recent report showed that low dose of one strain (*Bifidobacterium infantis* 35624, 1×10^{8} bacteria/capsule) reduced symptom scores in IBS patients,[8] and the result suggested that the effect of probiotics for IBS was dependent on a specific strain as well

as dose. Some strains of *Lactobacillus* were known to be effective in controlling IBS symptoms.[19] A paper studying an alteration of gut microbiota reported that *Lactobacillus* sequences were absent in stool from IBS patients, contrary to healthy control.[20] Regarding the reduction of abdominal pain by probiotics, a recent study demonstrated that one strain of Lactobacillus acidophilus induced the expression of mu-opioid and cannabinoid receptors in intestinal epithelial cells of rodents and mediated analgesic functions in the gut.[21] As described in introduction, considering the immunomodulatory effect of *Bifidobacterium bifidum* BGN4 and potential benefit of *Lactobacillus* sp. we selected study medication as composite probiotics composed of *Bifidobacterium bifidum* BGN4, *Bifidobacterium lactis* AD011, *Lactobacillus acidophilus* AD031 and *Lactobacillus casei* IBS041. All of them are original strains that were collected from Koreans and have been never used in clinical study.

We demonstrated that selected composite probiotics were effective in IBS patients (ITT population, n=70) as follows: pain reduced by 64% in probiotics group vs. 38% in placebo (p=0.045), and defecation discomfort reduced by 55% vs. 29% (p=0.043), respectively. In agreement to a previous report, probiotics were more effective in patient who excreted mainly normal or loose stool (baseline Bristol stool form scale $\geq$3, n=58) as follows: pain reduced by 66% in probiotics group vs. 29% in placebo (p=0.006), defecation discomfort reduced by 57% vs. 24% (p=0.013), and sum of scores reduced by 59% vs. 31% (p=0.010), respectively.[8] In contrast to many reports that probiotics showed minimal or no effect on abdominal pain, we demonstrated the beneficial effect in the treatment of IBS symptoms including abdominal pain using composite probiotics containing *Bifidobacterium bifidum* BGN4 and *Lactobacillus acidophilus* AD031.[6-8,16] Although Sinn *et al.*[22] reported that two strains of *Lactobacillus acidophilus* reduced abdominal pain by 20% more than placebo in IBS patients, we could demonstrate superior effect of probiotics on abdominal pain up to 37% over placebo. As compared with VSL #3 that showed effectiveness on overall score up to 16% over placebo, our composite probiotics were more effective on overall score up to 28% over placebo.[6]

χ^2-test was performed to test the response which was defined as reduction of symptom score by at least 50% after treatment in this study, but it could not show significant change. Data loss was inevitable in the process of converting symptom scores into responder status which was classified as yes or no, and it seemed to be the reason of low sensitivity of χ^2-test. By simultaneously doing parametric analyses over changes of individual scores after treatment, we could demonstrate the effect of probiotics accurately.

Although the analyses on bowel habits showed slight decrease of frequency, there was no statistically significant change between two groups, which might be due to low power of this study. A previous study showed normalization of frequency in *Bifidobacterium*-treated group through *post hoc* analyses (n=182) using data stratified by baseline bowel movements per day.[8] On the basis of further large-scale studies, probiotics are expected to be revealed as effective in correction of bowel habits.

In conclusion, composite probiotics containing *Bifidobacterium bifidum* BGN4, *Bifidobacterium lactis* AD011, *Lactobacillus acidophilus* AD031 and *Lactobacillus casei* IBS041 were safe and effective, especially in patients who excreted mainly normal or loose stool.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Health & Welfare, Republic of Korea (A060546-AD1101-06N1). Geun Eog Ji has the concurrent position in BIFIDO Co., Ltd. as well as a professor of Seoul National University.

REFERENCES

1. Everhart JE, Renault PF. Irritable bowel syndrome in office-based practice in the United States. Gastroenterology 1991;100:998-1005.
2. Talley NJ, Zinsmeister AR, Van Dyke C, Melton LJ 3rd. Epidemiology of colonic symptoms and the irritable bowel syndrome. Gastroenterology 1991;101:927-934.
3. Park KS, Ahn SH, Hwang JS, et al. A survey about irritable bowel syndrome in South Korea: prevalence and observable organic abnormalities in IBS patients. Dig Dis Sci 2008;53:704-711.
4. Chadwick VS, Chen W, Shu D, et al. Activation of the mucosal immune system in irritable bowel syndrome. Gastroenterology 2002;122:1778-1783.
5. Liebregts T, Adam B, Bredack C, et al. Immune activation in patients with irritable bowel syndrome. Gastroenterology 2007;132:913-920.
6. Kim HJ, Camilleri M, McKinzie S, et al. A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 2003;17:895-904.
7. Kim HJ, Vazquez Roque MI, Camilleri M, et al. A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. Neurogastroenterol Motil 2005;17:687-696.
8. Whorwell PJ, Altringer L, Morel J, et al. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. Am J Gastroenterol 2006;101:1581-1590.
9. Riedel CU, Foata F, Philippe D, Adolfsson O, Eikmanns BJ, Blum S. Anti-inflammatory effects of bifidobacteria by

inhibition of LPS-induced NF-kappaB activation. World J Gastroenterol 2006;12:3729-3735.
10. Park MS, Kim MJ, Ji GE. Assessment of lipopolysaccharide-binding activity of Bifidobacterium and its relationship with cell surface hydrophobicity, autoaggregation, and inhibition of interleukin-8 production. J Microbiol Biotechnol 2007;17:1120-1126.
11. Kim N, Kunisawa J, Kweon MN, Ji GE, Kiyono H. Oral feeding of Bifidobacterium bifidum (BGN4) prevents CD4(+) CD45RB(high) T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. Clin Immunol 2007;123:30-39.
12. Heaton KW, Radvan J, Cripps H, Mountford RA, Braddon FE, Hughes AO. Defecation frequency and timing, and stool form in the general population: a prospective study. Gut 1992;33:818-824.
13. Heaton KW, Ghosh S, Braddon FE. How bad are the symptoms and bowel dysfunction of patients with the irritable bowel syndrome? A prospective, controlled study with emphasis on stool form. Gut 1991;32:73-79.
14. Hays RD, Sherbourne CD, Mazel RM. The RAND 36-Item Health Survey 1.0. Health Econ 1993;2:217-227.
15. Patel SM, Stason WB, Legedza A, et al. The placebo effect in irritable bowel syndrome trials: a meta-analysis. Neurogastroenterol Motil 2005;17:332-340.
16. Williams EA, Stimpson J, Wand D, et al. Clinical trial: a multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study. Aliment Pharmacol Ther 2009;29: 97-103.
17. Dorn SD, Kaptchuk TJ, Park JB, et al. A meta-analysis of the placebo response in complementary and alternative medicine trials of irritable bowel syndrome. Neurogastroenterol Motil 2007;19:630-637.
18. Musial F, Klosterhalfen S, Enck P. Placebo responses in patients with gastrointestinal disorders. World J Gastroenterol 2007;13:3425-3429.
19. Camilleri M. Probiotics and irritable bowel syndrome: rationale, putative mechanisms, and evidence of clinical efficacy. J Clin Gastroenterol 2006;40:264-269.
20. Kassinen A, Krogius-Kurikka L, Mäkivuokko H, et al. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. Gastroenterology 2007;133:24-33.
21. Rousseaux C, Thuru X, Gelot A, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 2007;13:35-37.
22. Sinn DH, Song JH, Kim HJ, et al. Therapeutic effect of *Lactobacillus acidophilus*-SDC 2012, 2013 in patients with irritable bowel syndrome. Dig Dis Sci 2008;53:2714-2718.

International Journal of
Molecular Sciences

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Review

Review on *Bifidobacterium bifidum* BGN4: Functionality and Nutraceutical Applications as a Probiotic Microorganism

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Academic Editor: Alejandro Cifuentes
Received: 26 July 2016; Accepted: 8 September 2016; Published: 14 September 2016

Abstract: *Bifidobacterium bifidum* BGN4 is a probiotic strain that has been used as a major ingredient to produce nutraceutical products and as a dairy starter since 2000. The various bio-functional effects and potential for industrial application of *B. bifidum* BGN4 has been characterized and proven by in vitro (i.e., phytochemical bio-catalysis, cell adhesion and anti-carcinogenic effects on cell lines, and immunomodulatory effects on immune cells), in vivo (i.e., suppressed allergic responses in mouse model and anti-inflammatory bowel disease), and clinical studies (eczema in infants and adults with irritable bowel syndrome). Recently, the investigation of the genome sequencing was finished and this data potentially clarifies the biochemical characteristics of *B. bifidum* BGN4 that possibly illustrate its nutraceutical functionality. However, further systematic research should be continued to gain insight for academic and industrial applications so that the use of *B. bifidum* BGN4 could be expanded to result in greater benefit. This review deals with multiple studies on *B. bifidum* BGN4 to offer a greater understanding as a probiotic microorganism available in functional food ingredients. In particular, this work considers the potential for commercial application, physiological characterization and exploitation of *B. bifidum* BGN4 as a whole.

Keywords: *Bifidobacterium*; functional foods; probiotics; nutraceuticals

1. Introduction

The term functional foods and/or nutraceuticals can be defined as certain foods reserving bioactive compounds that likely have beneficial effects in the body beyond basal nutritional ingredients (i.e., carbohydrate, protein and fat) [1,2]. According to USA Food and Drug Administration (FDA) [3], "terms such as functional foods or nutraceuticals are widely used in the marketplace and these are regulated by FDA under the authority of the Federal Food, Drug, and Cosmetic Act, even though they are not specifically defined by law". Probiotics and probiotic foods are included within functional foods and have a growing market and large economic value [4]. The beneficial effects of probiotics on hosts beyond normal nutrition have attracted special interest from food industry and academia [5]. Functional properties of probiotic cells may offer various solutions to meet commercial demands for a variety of functional or conventional food products. Health benefits of probiotic cells coupled

with consumers' positive awareness regarding self-care, wellbeing and complementary medicine have reflected into multiple nutraceuticals as a promising ingredient in food industry [6,7]. Recently, multiple researchers have displayed interests to applications of food and nutraceutical processing to develop a novel concept of probiotic food or supplements [8,9].

Among the various probiotic bacteria, *Bifidobacterium*, is one of the most widely used and studied probiotic bacteria. According to Soto et al. [10], *Lactobacillus* and *Bifidobacterium* spp. accounted for 67.5% and 25.6% of microbial population, respectively, in breast milk (obtained from German and Austrian women, $n = 160$). Because the initial bacterial colonization is happening at an early stage of human life cycle, the primary colonization by breastfeeding or formula feeding has an important role to the individual health by affecting later host homeostasis during the development of the infant digestive and immune system [11]. Although, *Lactobacillus* is the major microbial flora in human milk, *Bifidobacterium* is the predominant cell species in fecal samples from breastfeed infants [12].

Naturally occurring microbiota in the intestinal tract of breast-fed infants, *Bifidobacterium* accounts for more than 80% of microorganisms within the intestine [13–15]. Among the various *Bifidobacterium* spp., *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* are commonly detected bacteria from breastfed infants [12], whereas formula-fed infants have a complex ecosystem comprising mostly of coliform bacteria and *Bacteroides*, with significantly lower prevalence of *Bifidobacterium* spp. [16]. For marketing purposes, some food researchers in industry have tried to develop infant formula that stimulates *Bifidobacterium* spp. to become the dominant flora by constituting bifidogenic factors (e.g., non-digestible carbohydrates, and galactooligosaccharides) [17].

Recently reported studies showed that *Bifidobacterium bifidum*, (*B. bifidum*) is the second most prominent species that identified in breast-fed infants (the first was *B. breve* and the third was *B. longum*) [18]. As an early colonizer of the infant gut, *B. bifidum* is widely present among fecal microbiota, however, the concentration of overall *Bifidobacterium* spp. is decreased during the progression of age while *B. adolescentis* and *B. catenulatum* reach greater levels in adult guts [19]. Individual results from multiple studies had a little variation, however, it was clear that *B. bifidum* is considered a dominant species of gut population in healthy breast-fed infants. This distinctive ecological feature of *B. bifidum* spp. attracted microbiologists' interests. Multiple experiments were carried out with clinical and pre-clinical studies, and proved significant health benefits (e.g., reducing bowel syndrome, diarrhea and pathogen infections) [20–23].

B. bifidum BGN4 (BGN4) *obtained from a* breast-fed *infant's* fecal sample came to the forefront in 1996 by its distinctive enzymatic representation: β-glucosidase (E.C 3.2.1.21) negative [24]. This microorganism was first used to evaluate the expression of mutagenic activity by β-glucosidase-producing gut microbiota that produce deglycosyl hydrolases and catalyze carcinogenic glycosides (i.e., amygdalin, anthrone-6-*O*-rhamnoside, 8-hydroxyquinoline-β-D-glucoside, neocycasin A, quercetin-3-*O*-rutinoside, guercitrin, robinin, and cycasine).

BGN4 has been applied to multiple nutraceutical products and conventional foods in the global food markets (e.g., China, Germany, Jordan, Korea, Lithuania, New Zealand, Poland, Singapore, Thailand, Turkey, USA, and Vietnam) as a probiotic microorganism because of possible benefits to consumers [25]. Multiple researchers have proven outstanding bio-functional characteristics of BGN4 by in vitro, in vivo, and clinical experiments. Potential benefits of BGN4 include: (i) notable colon cell binding properties [26,27]; (ii) improved immune function [28–34]; (iii) anti-tumor effects [27,35,36]; (iv) aid in bioconversion of phytochemicals [37–41]; and (v) production of biogenic metabolites [42,43].

These represent the five main findings that have been discussed with regard to functional benefits from BGN4 with in-depth research. Optimizing cell culture conditions could increase not only BGN4 cell biomass recovery but also its bioactive metabolites. Recently, BGN4 chromosome sequencing was completed and will therefore be analyzed to further understand the correlation between genetics and physicochemical properties [44]. This review will highlight the importance of each of the five areas. In addition, this review will address prominent prototypes of BGN4 products, distinguished genome

analysis and changed physicochemical attributes of BGN4 and the effect of altered culture conditions that were studied for the purpose of commercial manipulation.

2. Cell Adhesive Property

Foodborne illness (e.g., salmonellosis, listeriosis and shigellosis) occurs when food consumers eat a contaminated food with *Salmonella*, *Listeria*, and *Shigella* spp. [45]. Intestinal mucus and epithelial cells are predominantly susceptible to the attachment of pathogenic microorganisms resulting in active proliferation and colonization. This microbial adhesion is critical for the beginning of pathogen and epithelial cell interaction [46]. Consequently, avoiding bacterial adhesion onto the gastrointestinal mucosa is regarded as an efficient approach for decreasing the risk of foodborne disease [47,48]. Recently, Serafini et al. [49] observed inhibitory properties of *B. bifidum* PRL2010 against pathogenic bacteria (i.e., *Escherichia coli* and *Cronobacter sakazakii*) regarding enteric adaptation properties using epithelial intestinal cell monolayers (i.e., Caco-2 and HT-29).

Probiotic microorganisms are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by WHO and FAO. Among the many probiotic strains, *Lactobacillus* and *Bifidobacterium* spp. are known as autochthonous microbiota in the human intestinal tract. These microorganisms have been used in various functional foods for centuries. The major functional effects that are provided by probiotics are: (i) production of anti-microbial peptides (i.e., bacteriocins) [50–52]; (ii) assimilation of dietary fibers [53]; (iii) regulation of fat storage [54,55]; (iv) modulation of mucosal immunity [56]; and (v) regulation of gut flora via competitive exclusion of pathogenic bacteria resulting in decreased pathogen colonization [57–59]. Among the five key functional effects of probiotics, attachment of probiotic bacteria onto the mucosal surface of the gastrointestinal tract is regarded as essential for the competitive exclusion of pathogens and must occur before effective regulation of immune activities, resulting in protective function against intestinal pathogens [60,61]. The cell adhesion stage of probiotics onto colon cells is essential for the successful microbial colonization inside of the host's intestinal tract. This cell adhesion ability has been regarded as one of the critical screening standards for active probiotic strains [62], since adhesion is necessary to actively proliferate and provide a resistance to excretion from the intestinal tract as waste by peristalsis.

Mechanisms of bacterial adhesion onto epithelial cell can be divided into: (i) non-specific adhesion when regarding physicochemical factors of outer cell surfaces; or (ii) specific adhesion when considering the expression of specific molecules onto the microbial membranes that directly attach to the binding sites of epithelial cell mucosal surfaces [60–62]. This adhesion ability is a function of hydrophobic properties, level of ions, pH, and physical morphology [63]. These factors considerably affect microbial adhesion onto intestinal tissues of the host, demonstrating the complexity of preliminary microbial adhesion onto the mucosal surface.

According to Krasowska and Sigler [64], microbial hydrophobicity plays a key role in the initial interaction with the mucosal surface and epithelial cells of the intestinal lining due to the chemical composition of the bacterial surfaces. The physicochemical characteristics of the microbial outer membrane are generally estimated by analysis of cell surface hydrophobicity. It has been proven that microorganisms that express higher hydrophobicity more effectively attach onto the colon cells compared to hydrophilic microbial strains [61,62]. High cost and complexity of in vivo models encouraged attention into the use of an in vitro system for the initial selection and screening of potentially adherent probiotic microorganisms. Microorganisms that express high adhesive activity to inanimate surfaces (e.g., hydrocarbon surface) or non-polar solvents are considered hydrophobic, and cells that express lower adhesive activity are considered hydrophilic [62,64]. Pelletier et al. [65] reported that the existence of proteinaceous components on the microbial outer layer cause higher hydrophobicity, while hydrophilic properties are related to the existence of polysaccharides in the cell wall structure.

The hydrophobicity of strain BGN4 showed greater affinity towards xylene in similar studies (Table 1). Abdulla et al. [66] reported six different *Lactobacillus* strains with the hydrophobicity ranging from 29.5% to 77.4%. The three strains of *Lactobacillus* (i.e., *L. acidophilus*, *L. gasseri*, and *L. jensenii*) used by Boris et al. [67] showed about 80% surface hydrophobicity. *B. lactis* Bb12 and *L. acidophilus* LA5 showed surface hydrophobicity with values between 61% and 75% [68]. *B. pseudolongum* CIDCA 531 expressed 85% surface hydrophobicity [69]. Recently, Pan et al. [70] reported significant correlation between microbial adhesion property and cell hydrophobicity using 5 different *Bifidobacterium* strains (i.e., *B. longums* P-3, *B. animalis* H-9, *B. animalis* P-4, *B. asteroids* H-10 and *B. pseudocatenulatum* I-6) and Caco-2 with in vitro model. These results suggest that BGN4 may possess high cell adhesion properties and potent colonization abilities.

Table 1. Microbial hydrophobicity of the cellular surface (CHS) among reference strains.

No.	Cell	CHS (%)	No.	Cell	CHS (%)
1	*B. bifidum* BGN4	93	20	*B. longum* ATCC 15707	<5
2	*Bifidobacterium* KJ	90	21	*B. longums* P-3	18.5
3	*Bifidobacterium* HJ-30	90	22	*B. animalis H-9*	37.13
4	*B. adolescentis* ATCC 15703	90	23	*B. animalis* P-4	17.4
5	*B. animalis* ATCC 2552	86	24	*B. asteroids* H-10	49.5
6	*B. animalis* M6	85	25	*B. pseudocatenulatum* I-6	47.3
7	*B. animalis* Rd60	69.6	26	*B. pseudolongum* CIDCA	85
8	*B. animalis* SI	66.3	27	*B. lactis* Bb12	75
9	*B. animalis* CN2	21	28	*L. acidophilus* LA5	75.1
10	*B. bifidum* ATCC 2952	12	29	*L. paracasei* (lac 1)	80
11	*B. bifidum* RD54	7	30	*L. acidophilus* (lac 2)	65
12	*B. bifidum* MS1	6	31	*L. acidophilus* (lac 3)	60
13	*B. bifidum* SH5	6	32	*L. acidophilus* (lac 4)	30
14	*B. bifidum* E15	5	33	*L. fermentum* (lac 5)	45
15	*B. bifidum* E2-18	<5	34	*L. fermentum* (lac 6)	65
16	*B. bifidum* JS9	<5	35	*L. acidophilus*	80
17	*B. bifidum* SH2	<5	36	*L. gasseri*	80
18	*B. bifidum* SJ32	<5	37	*L. jensenii*	80
19	*B. infantis* ATCC 15697	<5	-	-	-

The data number 1 to 20, 21 to 25, 26, 27 to 28, 29 to 34 and 35 to 37 were adapted from Kim et al. [26], Pan et al. [70], Pérez et al. [69], Shakirova et al. [68], Abdulla et al. [66] and Boris et al. [67], respectively. The level of CHS was evaluated by the cell adhesive method into xylene.

However, we should point out that the hydrophobic surface characteristics of probiotic bacteria do not consistently bind to epithelial colon cells [71,72]. The distinguished physicochemical surface of probiotics do not guarantee binding to epithelial colon cells. The adhesion property of microorganisms is significantly inconsistent and heterogeneous among cell strains [73]. Specifically, some probiotic strains show effective cell adhesion ability although they express significant hydrophilic properties on their cell surface [74]. This shows that other aspects that affect cell adhesion should also be considered. To overcome the limitation of cell hydrophobicity that often accompanies adhesion ability, microbial adhesion experiments using in vitro models with intestinal epithelial cells have been extensively *i*nvestigated [26,27,44,62,73]. The number of microorganisms attached to culture tissues directly shows the cell adhesion property. Among the various intestinal epithelial cell lines, the enterocyte-like Caco-2 cells obtained from a human colon have been routinely used to examine microbial adhesion mechanisms because of their distinctive physicochemical characteristics (i.e., active proliferation and differentiation under normal enrichment conditions, similar biological characteristics to normal enterocytes) [75].

BGN4 was compared to twenty different strains of *Bifidobacterium* spp. (i.e., *B. bifidum*, *B. animalis*, *B. adolescentis*, *B. infantis* and *B. longum*) separated from human fecal samples to evaluate cell adhesion properties [26]. According to Crociani et al. [76], cell adhesive properties of Bifidobacterium spp. are highly variable between strains of the identical genus. Kim et al. [26] clearly illustrated that binding between BGN4 whole cells and well-defined brush border microvilli on Caco-2 using scanning electron microscope (SEM). Among the various strains of *Bifidobacterium*, BGN4 showed the largest number of cells bound to the Caco-2 cells with highest cell surface hydrophobicity (93%) (Figure 1). Recently,

2.2 Mb of the BGN4 genome sequence was completely decrypted [44]. The comparative genomic analysis clearly elucidated the existence of a homolog (BBB_0596) of the *B. bifidum* MIMBb75 outer protein (BopA) that aids in the sticking of microorganisms onto a Caco-2 cell layer [44].

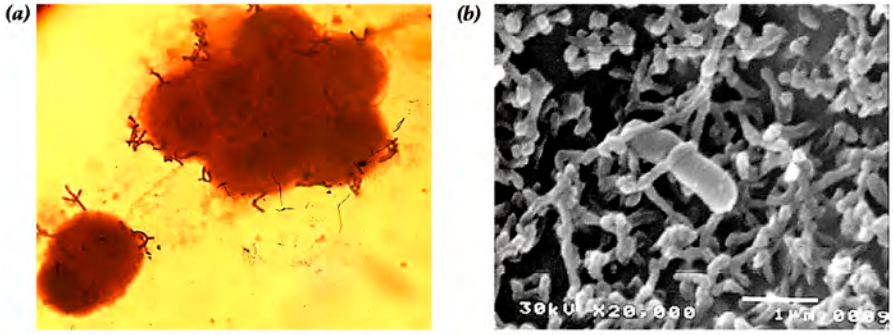

Figure 1. Adhesion of *B. bifidum* BGN4 onto the epithelial Caco-2 cell observed by: (**a**) optical (magnification of 1000×); and (**b**) scanning electron microscopy (magnification of 20,000×, interaction with microvilli of Caco-2 and *B. bifidum* BGN4). Microbial adherence in (**a**) was observed after simple staining with crystal violet. Panel (**b**) was adapted from Kim et al. [26].

Despite this, very little research was done on specific adhesion related to the molecular mechanisms which possibly affects the strong adhesion properties, overall these findings evidently demonstrated the notable cell adhesive ability of BGN4 onto epithelial cell with its high hydrophobicity under in vitro conditions, and thus could represent better ability to colonize in the gastrointestinal tract with protracted transit. More detailed understanding of the: (i) adhesive mechanisms of BGN4 under the molecular level; (ii) fecal samples; and (iii) intestinal lining by biopsies could allow us to know the significance of adhesive ability of BGN4 and its applications to functional foods.

3. Immune-Modulatory Effects of *B. bifidum* BGN4

Multiple probiotic strains have shown significant bio-functional properties concerning boosted host immune functions. One of the important roles of probiotic bacteria is immune-modulatory activities for the prevention and regulation of multiple enteric diseases in the host [77]. According to Galdeano [78], orally consumed fluorescent-labeled probiotic cells were identified in the immune system (i.e., Payer´s patches and lamina propria mucosa) in the small intestine and lymphoid tissues (i.e., lymph nodules and colonic crypts). This report provides convincing evidence of a direct interplay between probiotic microorganisms and immune cells in the host's intestinal lining.

Among the various immune cells, phagocytic cells (i.e., neutrophils, monocytes and macrophages) in the intestinal mucosa play an essential role in both stimulation of inflammatory responses against potential enteric pathogens and tolerance of normal colonic luminal nutrients and microbes as an innate immune system [79]. When macrophages are under an inflammatory stimuli, they generate cytokines, including Interlukin (IL)-1, IL-6, IL-8, IL-12, and tumor necrosis factor (TNF), which recruits other inflammatory cells. Phagocytic cells are attracted toward specific infection sites to engulf the opsonized targets using phagocytosis. They recognize pathogens using chemotaxis stimuli and/or straight physical connections [80–82]. Multiple reports have shown a significantly promoted phagocytic capacity of phagocytes by probiotic supplementation as an immunomodulator [83]. Therefore, the evaluation of the level of cytokines and macrophage activity using an in vitro assay is considered an indirect way of analyzing bio-functional effects of probiotic cells.

Lee et al. [28] reported the significant immunoregulatory capacities of whole-cell and cell-free extracts derived from BGN4. In this work, when macrophages were exposed to BGN4, active cell division, greater cytokine production and active phagocytic property were observed. Since then, various studies have focused on the interactions between outer cell wall and immune cells, however,

little work that employs intercellular ingredients has been reported before. Therefore, they also extracted four different BGN4 cell fractions (i.e., whole-cell, cell free extracts, purified cell wall and supernatant) and treated cell lines to evaluate the level of cytokine produced by macrophages. As a result, each fraction showed different patterns of immune reactions. The whole cell fraction represented the strongest TNF-α expression. The cell-free extracts of BGN4 induced the highest IL-6 production.

This work was confirmed and further explained by Kim and Ji [29]. They made an attempt to determine the significance of type of BGN4 cell fractions with special focus on location within the host immune system. All BGN4 cell fractions (i.e., cell free extracts, whole cell fractions and cell wall fractions) significantly stimulated the production of IL-10 and IL-6. Cell free extracts of the BGN4 were able to induce greater morphological modification of macrophages with increased phagocytosis properties compared to macrophages treated with other BGN4 fractions (i.e., whole cell and cell wall fractions). The use of an in vitro assay clearly showed the immunomodulatory properties of BGN4 that activate differentiation of macrophages.

Another experiment was performed to examine the immune responses of intragastrically administrated BGN4 in a murine model of peanut allergy to provide further support function of BGN4 based on in vivo experiments [30]. They concluded that BGN4 treatment in an animal model showed anti-allergic and immunomodulatory effects by decreased levels of peanut-specific IgE and IL-4 and increased levels of IL-12 and the ratio of Interferon (IFN)-γ/IL-4. Kim et al. [31] also reported clinical properties of BGN4 to inflammatory bowel disease using a mouse model. The BGN4-fed group showed minimal signs of thickened wall and inflammatory cell infiltration, in a clinical sense, such as: (i) thickened wall; (ii) crypt elongation; (iii) reduction of goblet cells; and (iv) maintaining the level of cluster of differentiation (CD) 69, IFN-γ, TNF-α and MCP-1 in the mouse intestine than its counter group. The in vivo approaches employed in this work clearly suggest further functional characterization of BGN4 on the control of the aberrant intestinal immunity.

However, an additional question is: does BGN4 show potent immune stimulating effects within clinical experiments? This answer is critical to prove practical benefits of BGN4. Despite various in vitro, in vivo data, the precise mechanism of action of BGN4 was not fully demonstrated and could be multifactorial in clinical research.

According to Hong et al. [32], a probiotics mixture containing 2×10^{10} of lyophilized cells (i.e., BGN4, *B. lactis* AD011, *L. acidophilus* AD031and *L. casei* IBS041) was effective to relieve irritable bowel syndrome. They randomly divided two groups (n = 36 and 34; age: 19–75 years; sex: male and female; symptoms: presence of previous gastrointestinal disease) as probiotics and placebo groups, respectively. Their work clearly demonstrated that probiotics treatment was statistically significant in the reduction of abdominal pain and defecation discomfort after eight weeks of probiotics treatment compared to placebo groups (n = 70, −31.9 vs. −17.7, p = 0.045), and concluded "composite probiotics containing BGN4, *L. acidophilus* AD031, and other species are safe and effective, especially in patients who excrete normal or loose stools".

Kim et al. [33] used different strategies to characterize functional effect of BGN4 for their role in eczema. Through the randomized, double-blind and placebo-controlled experimental design (n = 112, screened and randomized pregnant women having family history of allergic diseases), they evaluated the preventive function of BGN4 against progress of eczema. They concluded that the prevalence of eczema can be statistically significantly decreased by BGN4 treatment compared to its counter placebo group (completed sample number: n = 68, p = 0.048, BGN4 group: 18.2% vs. placebo: 40.0%). Interactions among gut microbiota, intestinal epithelial cells and mucosal dendritic cells in the lamina propria, and their impact in innate immunity has been the focus of multiple researchers in recent decades [84]. Specifically, Kim et al. [34] discussed function of BGN4 treatment into the dendritic cells. With comparison of cell culture conditions (i.e., single culture of dendritic cells or co-culture of dendritic cells and mouse epithelial cell monolayers), multiple conditions for exerting immune-modulatory reactions were evaluated. The authors concluded that BGN4 significantly upregulated the expression of I-Ad and cluster differentiation (i.e., CD86 and CD40) ($p < 0.05$) with increased secretion levels

of pro-inflammatory cytokines (i.e., IL-6 and TNF-α). These results indicate that BGN4 potentially stimulates immune modulation via interaction of dendritic cells in the gut homeostasis.

As noted above, there are multiple experiments that provide evidence that BGN4 potentially affects the host immune systems and exerts protective actions from allergens through in vitro and in vivo studies, and show promising advances in the application of nutraceutical fields. Accumulating results indicate that some symptoms that are triggered by artificially treated allergens or antigens can be relieved by BGN4. Although physiologic outcomes have suggested possible benefits of BGN4, clinical efficacy of BGN4 has not been clearly established using single type of cell ingredient. Therefore, available evidence is not enough to demonstrate whether BGN4 may be more effective for bio-functionality in the human body than other microorganisms. Interpretation of the functional evidence of BGN4 is hampered by the presence of numerous other microorganisms. Further physiologic investigations are necessary to design formulations and to understand the basic mechanisms and bioavailability for studies of physiologic actions using single type of BGN4.

4. Anticancer Effects of *B. bifidum* BGN4

Colorectal cancer is a global health issue; in particular, South Korea showed the highest number of colon cancer cases in the world. As the third leading type of cancer in the world, approximately 1.4 million cases were identified in 2012 [85]. According to the American Cancer Society [86], colon cancer is the second leading cause of cancer mortalities with an expected 49,190 deaths in 2016. The international incidence and mortality rates of colorectal cancer are rapidly increasing in multiple countries as eating habits have been altered to a more occidental manner (i.e., low-dietary fibers, high-fat and high-protein) [87].

Various studies have reported that fermented food products can significantly prevent tumor growth by decreasing the risk of long-standing inflammatory responses in colon cancer. Probiotic microorganisms normally contained in fermented food products are known to offer functional effects on mucosal damages, specifically preventing the effects of cancer on the digestive tract [88]. Probiotics have multiple therapeutic advantages, playing significant roles in decreasing the mutagenicity of the epithelial layer, as reported in various experimental models of colorectal cancer [56]. In-depth investigations have shown that the relationship between colorectal cancer and probiotics seems to be primarily dependent on bioactive metabolites of probiotic bacteria, which lead to the generation of therapeutic anti-carcinogenic compounds [89].

Certain probiotic microorganisms and their extractions demonstrate growth inhibitory activities on adenocarcinoma cell lines. In particular, fractions from *Bifidobacterium* and *Lactobacillus* spp. containing high levels of microbial carbohydrates (i.e., extracellular glycoproteins, peptidoglycan, and polysaccharide) displayed profound tumor-suppressing activities [90–92]. These studies have demonstrated on evaluating the properties of probiotic fractions and extractions with regard to the decrease of viability or size of cell lines. However, proving the selective inhibition on cancer cells by probiotics treatment is critical with regard to the screening and selection of anti-carcinogenic substances. Moreover, anti-carcinogenic properties of probiotic microorganisms on colorectal cancer significantly differ from strain to strain, making it necessary to screen novel probiotic strains for tumor inhibitory effects [93]. Therefore, researchers should attempt to study the selectivity, sensitivity and specificity on noncancerous as well as cancerous cell lines using multiple probiotics.

Microbial polysaccharides are produced by probiotic bacteria with various health-promoting effectiveness. Their chemical structures, complexity and molecular weights differ among the probiotic species, resulting in expression of different physicochemical characteristics in both in vitro and in vivo systems. The antagonistic properties of probiotic bacteria against gastrointestinal illneses have been the subject of many clinical investigations, demonstrating varied functional properties. According to Nagaoka et al. [94], probiotic polysaccharides extracted from *B. breve* YIT4014 and 4043, and *B. bifidum* YIT4007 have shown anti-ulcer activities both directly (i.e., epidermal and fibroblast growth factor) and indirectly (e.g., immune system stimulating: increased production

of 6-keto-prostaglandin F1 α by macrophage). Specifically, polysaccharides containing rhamnose as a major content (more than 60%) showed greater effectiveness in healing gastric ulcers. The soluble polysaccharides produced from *L. acidophilus* 606 also expressed the inhibitory effects on progression of colorectal cancer cell lines such as HeLa, PANC-1 and HT-29 cells and partially induced apoptosis into the HT-29 [90]. However, the polysaccharides from *L. acidophilus* 606 exhibited minimum toxicity into healthy human embryo fibroblasts.

These findings provided motivation to our group for observation and hypothesis on the correlation between BGN4 treatments and human colon cancer. Ku et al. [27] and You et al. [36] have demonstrated the tumor-suppressing activity of whole cell and its fractions of BGN4 on diverse adenocarcinoma cell lines. They also attempted to clarify whether such properties were human colon cancer cell-specific. Among the 30 kinds of different strains of *Bifidobacterium* tested in these studies, BGN4 showed the greatest anti-proliferative effects on human colon cancer cell lines. In particular, the polysaccharide fractions comprising chiroinositol, rhamnose, glucose, galactose, and ribose that were extracted from BGN4 induced significant growth inhibition of cancer cell lines (i.e., HT-29 and HCT-116), but did not show any growth inhibition of FHC (normal human colon cell) or Caco-2 cells, which are generally used as control group because of similar physicochemical properties with normal cells [95].

Previously, Shim et al. [35] discussed health benefits of fermented soy milk by BGN4, and confirmed that dietary BGN4 decreases the size of azoxymethane-induced aberrant crypt foci in rats. The combination of soymilk and BGN4 showed significant synergistic effects on reducing the number of aberrant crypt foci. The size and number aberrant crypt focus are commonly used biomarkers for colorectal cancer in rodents [96] and often regarded as the earliest histopathologic lesion linked to colon cancers [97].

The simple assessments of a decreased cell proliferation and aberrant crypt foci levels using in vitro and in vivo experiment are not enough to evaluate anti-cancer and/or anti-tumor properties of BGN4 due to the complexity of cancer development, which is linked to numerous cellular mechanisms. However, the anti-proliferation properties of BGN4 onto multiple cancer cell lines could be used as an example of the interaction between *Bifidobacterium* spp. and host. The interactions between BGN4 and colon cancer stimulated novel manners of cancer suppression and suggested the treatments of cell extractions with probiotic substances for the purpose of gaining anticancer properties. Further mechanistic studies and human epidemiological studies are necessary to elucidate the role of BGN4 and its extractable polysaccharides as a therapeutic option for anticancer or antitumor effects using animal models to take advantage of clinical properties derived from BGN4.

5. Industrial Application: Biocatalysis

Applications of health benefits from probiotics depend on the production of functional cell metabolites [5,7,52,56]. The commercial significance of health beneficial metabolites (i.e., polyssacchrides, bacteriocine, γ-Aminobutyric acid (GABA), and *S*-Adenosyl-L-Methionine (SAM)) from probiotic bacteria has stimulated the use of these bacteria as "Biological Factories" of value added products [98]. Bio-functional metabolites are produced by a variety of probiotic cells, especially *Lactobacillus* and *Bifidobacterium* spp., and have been intensively studied due to their broad spectrum of bio-functional properties and beneficial roles in human body. Due to the significance of probiotic cells in the expression properties of functional molecules, probiotic cells have been used in industry manufacturing for the production of value-added molecules [7,9,21,33,75].

Normally, the claimed functional benefits are likely achieved with high level of probiotic cells, however, multiple studies showed poor nutraceutical availability of some probiotic microorganisms in functional food products and found that they often exist at lower concentration in cells than those claimed on product packages [99]. Due to severe food processing and storage conditions (e.g., heat and acid treatment, artificial and natural preservatives, freeze and osmotic shock, and oxygen stress) often applied during the manufacturing step, maintaining cell activity and viability are practical challenge. Moreover, during the long-term period of circulation, viability of probiotic cells in product does not

meet the criteria at the end of shelf life. Therefore, it is necessary to maintain cell viability and ensure probiotic effect for consumers' needs [100]. A simple addition of probiotic cells into foods cannot guarantee health benefits to consumers. Therefore food industries have also encountered a number of difficulties when claiming the functional effects on the package of food products. For this reason, industry researchers and marketers have pursued to explore more applications of probiotics that can potentially be utilized in industry geared at several different markets [99]. Recently, various studies have proposed to use probiotic immobilization techniques to maintain microbial functionality and viability [101].

Extensive attention has been paid to the potential of using whole cell and/or cell fractions to facilitate the production of functional molecules [7,36,102]. Food and biotechnology industries have used advances in probiotics and their enzymes to produce value-added plant metabolites and/or their chemically transformed substances. Recently, bio-functional potentials of traditional herbal medicines and normal plants have emerged, resulting in notable progress in commercial developments of functional foods and/or nutraceuticals [41,102–106]. Specifically, to improve the quality of herbal resources, multiple probiotic cells and their enzymes have been applied for decades with commercial and domestic purpose in Korea under the concept of "fermented plant medicine" in the development and launch of nutraceuticals that is conceptually differentiated to other products [9,107].

This trend can be explained by favorable images of probiotics and herbal medicines among Korean consumers. According to Siró et al. [1], "Consumers need to understand the benefits, not the science behind the product". Because of consumers' limited understanding of functional foods and their health benefits, use of novel bio-functional materials could generate unnecessary work load and marketing cost to advertise and inform specific functional effects to consumers [99].

Phytochemicals are biologically and nutraceutically valuable plant metabolites. The isolation and recovery of target natural products from plants is often available for small quantities, specifically when small amounts of target molecules are naturally produced by plants and preserved in them [108]. Therefore, artificial pre-treatment (i.e., physical, chemical and enzymatic treatment) of phytochemicals to modify their chemical structures results in an increased yield of bioactive molecules and has conventionally been used to overcome limited supply issues. According to Gao et al. [107], biotransformation is "a chemical reaction that is catalyzed by whole cells (microorganisms, plant cells, animal cells), or by isolated enzymes due to high stereo- or regioselectivity combined with the high product purity and high enantiomeric excesses". Bioavailable plant metabolites, specifically when they are in a glucoside form, are known to be functionally fortified by a deglycosylating process [9]. This biotransformation process selectively hydrolyzes target molecules and enables the structural conversion into valuable products. Biotransformation using biological catalysis can be carried out under relatively mild operational conditions compared to physical (heat treatment) reaction and/or chemical (i.e., acid and basic) catalyst counterparts, abridging the multifaceted manufacturing process [108–110]. Recently, biotransformation utilizing catalytic activity of microbial glycosidases has been recognized as useful technology in nutraceutical and pharmaceutical industries. Specifically, biotransformation of phytochemical glycosides using probiotic glycosyl hydrolases has played a great role in the production of bio-functional phytochemical aglycones with attractive potential for practical applications [102]. This bio-catalytic process using probiotic enzymes has been studied and applied as an essential manufacturing tool for enhancing the bio-functional and nutritional values of herbal medicines [107]. Through the fermentation process, plant glycosides can be catalyzed to aglycone, which has better bio-functional effects. Probiotic whole cells and their extracts (i.e., purified enzyme, cell-free extracts and crude homogenates) are increasingly utilized in the nutraceutical industry as key ingredients [9]. They have also been used as bio-catalytic agents that play a fundamental role in the bioconversion of herbal glycosides into aglycones induced by microbial enzymes belonging to different groups of glycosidases.

Panax ginseng, meaning "cure-all", and its major functional metabolite, ginsenosides, are one of the widely-researched herbal medicines and phytochemicals [109]. There are multiple

studies available covering various pharmaceutical properties of ginsenosides. In accordance with molecular mechanism, various pre-clinical and clinical studies have suggested that the regulatory properties of deglycosylated ginsenosides on diverse cellular mechanisms (i.e., cell cycle regulator (cyclin-dependent kinase), transcription factor (myc gene), signal protein (vascular permeability factor), tumor suppressors (cellular tumor antigen p53), cyclin-dependent kinase inhibitor (CDK-interacting protein 1), negative regulator of the p53 (mouse double minute 2 homolog) and apoptosis regulators (B-cell lymphoma-extra large, B-cell lymphoma-2 and X-linked inhibitor of apoptosis protein, etc.)) may have the anti-carcinogenic ability in prevention and management of cancer and chronic diseases [102]. Many studies have reported the phytoestrogenic properties of soy milk and soy isoflavones. Multiple evidences have shown that soy-derived phytoestrogens play inhibitory roles in osteoporosis, obesity and diabetes [111–114]. However, the functional effect of ginsenosides and soy isoflavones in in vivo systems are significantly dependent on enzymes of gut flora [115]. Therefore, merging probiotics and these phytochemicals may improve beneficial effects associated with intake of this plant medicine. Daidzein, genistein and glycitein that deglycosylated from of daidzin, genistin and glycitin have been introduced as chemo-preventive compounds for certain types of cancer (i.e., colon, breast and prostate) [116] and osteoporosis [117]. Similar to glycosylated ginsenosides, multiple studies demonstrated that the soy isoflavones in plants should be catalyzed into deglycosylated form (i.e., daidzein and genistein) for the effective absorption into blood stream across the gastrointestinal tissue [118]. The higher bioavailability after deglycosylation process has been demonstrated in in vivo experiments and explained by lower molecular weight and greater hydrophobicity than those of glycosidic compounds.

Because BGN4 naturally does not produce β-glucosidase during fermentation, soy isoflavones and ginsenoside glycosides cannot be catalyzed into the more bio-functional aglycones [24]. In this sense, BGN4 had practical limitation for industry application. Some researchers have manipulated expression vector to produce the recombinant BGN4 strain by cloning the structural β-glucosidase gene from naturally β-glucosidase producing *Bifidobacterium* spp. (i.e., *B. lactis* AD011, *B. lactis* SH5 and *B. lactis* RD68) [37–41]. β-glucosidase of *B. lactis* AD011 was cloned and overexpressed to apply ginsenoside conversion by Kim et al. [38]. BGN4 was employed as a sub-cloning and overexpression host for cloning of β-glucosidase of *B. lactis* AD011. However, BGN4 transformants (B141 and B893) could not use to catalyze artificial substrate (pNP-β-D-glucopyranoside) as well as natural substrates (ginsenosides). To attack this problem, Wang et al. [39] have attempted to highlight the necessity of powerful promoters for BGN4 in inducing significant expression of cloned genes with special focus on an exploration of the activity configurations of the promoters in BGN4. They proved that the activities of promoters were a function of microbial growth rates and that the use of P919 is effective to express of high-levels of foreign genes as a BGN4 promoter by hydrolyzing pNP-β-D-glucopyranoside into *p*-nitrophenyl and glucose. However, it should be considered that microbial β-glucosidases are quite unspecific catalysts whose specificities and activities possibly depend on a structural diversity of glycosides.

Recently, Youn et al. [37] highlights further how genetically-transformed BGN4 can be characterized as a β-glucosidase producer to practically apply for hydrolyzing natural products in which sugar moieties are linked to functional groups by a glycosidic bond (glycosides). They have constructed multiple expression vectors systems using bifidobacterial promoters (i.e., pamy, p919 and p572), ORF (i.e., bbg572), terminator (i.e., 572t) and signal sequences (i.e., ssamy) to produce new recombinant β-glucosidase-positive BGN4. The recombinant β-glucosidase, Bp572bbg572t was applied to catalyze multiple disaccharides (i.e., cellobiose, sophorose, laminaribiose and gentiobiose), Isoflavones (i.e., daidzin, genistin, and glycitin), ginsenosdies (i.e., Rb1 and Rb2) and Quercetins (i.e., isoquercetrin and spiraeoside) and successfully degraded glycosidic linkages between two molecules. This work would be practically useful for an application in phytochemical and bioconversion industries due to the understanding the vector systems/enzyme functions relationship. You et al. [41] also successfully carried out enzymatic catalysis of the isoflavone glycosides (i.e., daidzin,

genistin and glycitin) into isoflavone aglycones (i.e., daidzein, genistein and glycitein) using a novel recombinant β-glucosidase. The β-Glu gene of *B. lactis* AD011 consisting of 1.4 kb was cloned and the recombinant β-glucosidase was overexpressed in BGN4.

As a result, BGN4 notably produced β-glucosidase and could be applied to convert ginsenoside glycosides and soy isoflavones into deglycosylated forms. The catalysis of plant glycosides using recombinant BGN4 would be applicable for commercial purposes [111,115]. The combination of this recombinant BGN4 with plant compounds could be utilized to produce fermented plant medicines with elevating amount of bioactive forms of ginsenosides and isoflavones. In addition, the synergistic effects generated by indigenous functional properties of BGN4 and its biogenic metabolites can be possibly expected. Recently, 110 Nobel Prize winners from diverse domains (i.e., medicine, economics, physics, chemistry, literature and peace) issued a statement in their support of modern genetic engineering, such as GMOs [119]. However, resistance to genetically-engineered probiotics from food consumers may exist in food market, resulting in the search for a new market (i.e., biomedical and pharmaceutical markets) exploitation beyond food or nutraceuticals, and consumers' paradigm shift seems be necessary for successful commercial applications [1,9]. Before extensive utilization of genetically tailored BGN4 in nutraceutical products, consideration of possible safety issues and consumers' prejudice is necessary.

6. Industrial Application: Bioactive Molecules

Among the various functional cell metabolites, GABA, a ubiquitous non-protein amino acid, is widely present in natural resources including bacteria, plants, and animals [8]. This molecule acts as a major inhibitory neurotransmitter in the brains of vertebrates and is produced by α-decarboxylation of glutamate by glutamate decarboxylase [120]. Recently, multiple studies have reported bio-functional effects of GABA (i.e., hypotensive, energy boosting, tranquilizing, lessens signs of aging, diuretic effects and anti-diabetes) [120,121]. Microbial glutamate decarboxylase, which is critical in GABA production, is widely distributed in probiotic cells. Multiple *Lactobacillus* spp. have expressed an ability to produce GABA in various levels depending upon the density of glutamates in the cell culture broth [122,123]. Recent reports have shown that *Gastrodia elata*, a traditional Asian plant medicine often applied for the treatment of neurodegenerative diseases and headaches, represses the degradation of GABA [124] and protects against neuronal damage. As a raw material, *Gastrodia elata* is regarded as a useful raw material for the GABA production due to the synergistic anti-hypertensive functions originated from *Gastrodia elata* [125].

To develop fermented *Gastrodia elata* products containing considerable amount of GABA, Kim et al. [30] applied *Lactobacillus brevis* GABA 100 and BGN4 simultaneously as a starter culture for *Gastrodia elata* fermentation. Previously, Kim et al. [126] reported high GABA-producing properties of *Lactobacillus brevis* GABA 100 that is isolated from Korean kimchi. The total GABA productivity was further increased by the co-culture of *L. brevis* GABA 100 with BGN4. The level of GABA observed during the co-culture was higher compared to the culture inoculated only by *L. brevis* GABA 100 by further decreasing media pH compared to its decrease in the single culture of *L. brevis* GABA 100. According to Komatsuzaki et al. [127], maintaining low pH (about 5) is necessary for effective GABA production. However, during the fermentation, normally the pH level of the cell culture media increased due to the enhanced level of GABA in the media.

As discussed above, due to the bioactive functionality of probiotic bacteria and its value-added metabolites, interest in mass production and industrial applications of biogenic molecules has been growing. Specifically, SAM, a commercially available and FDA-approved dietary supplement, has often been obtained and produced through chemical synthesis and cell fermentation. However, chemical synthesis has issues with production cost and generation of low purity products with optical isomers [128]. SAM, which is an amino acid naturally produced in the human body, plays a key role in transmethylation as a methyl donor. Multiple studies have extensively revealed the functional effects of SAM. As an important nutraceutical ingredient, SAM showed anti-depressant [129], anti-liver

disease [130], and anti-headache effects [131]. According to Kim et al. [43], BGN4 produced a higher level of SAM compared to other microorganism. They used 25 kinds of different lactic acid bacteria (i.e., *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus* and *Weissella* spp.) and evaluated the productivity of SAM in culture media. The SAM productivity of BGN4 was at least two times higher than other bacterial strains. They also applied BGN4 to develop SAM-reinforced yogurt and reported favorable sensory value for commercial purposes [42]. However, little work was done to elucidate how productivity of above mentioned metabolites may be influenced by environmental factors including media ingredients, temperature and presence of other bacteria for commercial purposes. Nonetheless, above mentioned experimental data supports that BGN4 can be used in nutraceutical products as a microbial ingredient due to the benefits for human health.

7. Increase Biomass Productivity

For the commercialization, estimating productivities of cell and/or biogenic metabolites is necessary for cost-effectiveness of product manufacturing. In this sense, determination of appropriate media ingredients and formula design are important to enhance total cell-biomass productivity, as both biochemically and physiologically affect probiotic cultures [132]. When considering production costs of cell or biogenic molecules for commercial application, microbial enrichment for biomass production can be halted at the time of maximum productivity and some are left to run for longer, depending on the culture condition [133]. However, there are several pragmatic obstacles in mass production *Bifidobacterium* spp. and its metabolites for commercialization due to: (i) lower cell productivity after enrichment; and (ii) higher production cost compared to other aerobic or facultative anaerobic cells.

Kwon et al. [134] worked to develop a strategy to obtain high BGN4 biomass with greater metabolic products by combination of crossflow filter and cell reactor. Specifically, they submerged hollow fiber membrane (0.4 μm cut off, polyvinylidene fluoride, surface area of 25 m^2) bioreactor with suction and gas sparging to maintain anaerobic conditions. By using this method, they were able to observe higher BGN4 viability and lower microbial harms generated by shear stress during crossflow filtration processes compared to conventional membrane reactor culturing. About 5 and 7 folds greater productivity of BGN4 cell biomass and viable cell counts (i.e., 12.0 g/L of biomass productivity and 2.2×10^{10} CFU/mL of maximum cell count) were observed when submerged hollow fiber membrane bioreactor was applied for BGN4 enrichment compared to the microorganism levels achieved through conventional batch culture (i.e., 4.5 g/L of biomass productivity and 3.0×10^9 CFU/mL of maximum cell count).

Recently, Ku et al. [27] and Ji et al. [135] have reported more systematic approaches to increase BGN4 biomass productivity. They examined multiple factors, including: (i) media ingredients; (ii) types of acid; and (iii) incubation time when they utilized a two-step culture method that significantly affects the total recovery of BGN4 biomass and its bioactive metabolites. They reported that the phytic acid in culture media plays important role in improving the productivity and economic of BGN4 cell biomass and its biogenic molecules by changing microbial morphology and increasing size of the cell, although additional phytic acid treatments constitute a small portion from the overall costs of culture media formulation. These results agreed with various results showing that microbial shape and morphologies increase in the group in which media was treated with supplemented acids compared with those of control group [136–139]. It seems that the putative morphological modification effects of organic and mineral acids are likely due to their ability to act as cation chelators to induce response of the microbial mesosome control [140].

Even though multiple researchers have observed the putative role of acids as major inducer for the morphological modification of various bacteria, the molecular mechanisms underlying the producing properties of cell biomass are still largely unknown. Because chromosome sequencing data is available for BGN4, this information can be potentially utilized for designing better conditions of the biomass recovery than is currently possible. Moreover, transcription profiling over the fermentation procedures will provide information for stress and acid tolerance genes for BGN4. Additional work is necessary to

better understand how genetic characteristics of BGN4 affect the recovery and production of BGN4 and its bioactive molecules using post-genomic approaches.

8. From Comparative Genomics to Functionality of BGN4

The *Bifidobacterium* genus is currently comprised of 47 recognized taxa, which have been isolated from six different ecological environments including the gut and oral cavity of human and animals insect hindgut, sewage and fermented foods [141–143]. The *Bifidobacterium* taxa can be clustered into six different phylogenetic taxa: *B. adolescentis*, *B. longum*, *B. pseudolongum*, *B. boum*, *B. pullorum*, and *B. asteroids*groups [144]. Although *B. bifidum* species have been represented one of the dominant bacteria from the gastrointestinal tract of breast milk-fed infants, *B. bifidum* is not included in six phylogenetic groups mentioned above, suggesting its unique and specific genomic composition [19,145].

The publicly available genome sequences to date contain three complete genomes of *B. bifidum* strains obtained from infant stool samples including BGN4 and 12 draft genome sequences (NCBI source). Among the genus *Bifidobacterium*, 23 complete bifidobacterial genome sequences are available. The size of *B. bifidum* genome is approximately 2.2 Mb (range, 2.14–2.28 Mb) and GC content is about 62% (Table 2).

Table 2. Publicly available genome datasets of three different *B. bifidum* strains.

Strain Name	*B. bifidum* BGN4	*B. bifidum* PRL2010	*B. bifidum* S17
Accession	NC_017999.1	NC_014638.1	NC_014616.1
Sequencing Status	Complete	Complete	Complete
Genome Size (bp)	2,223,664	2,214,656	2,186,882
G + C ratio (%)	62.65	62.67	62.76
Number of Chromosones	1	1	1
Number of Contigs	1	1	1
Number of ORFs	1834	1706	1783
Number of rRNA Genes	9	9	9
Number of tRNA Genes	52	52	53

In accordance with the analysis of other bifidobacterial taxa, enzymes in charge of the transport and metabolism of carbohydrates (Clusters of Orthologous Genes, COG category, G) were identified from the genome of BGN4 (Table 3), such as glycosyl hydrolases whose substrates are various oligo- and polysaccharides including human milk oligosaccharides (HMOs) and intestinal mucin [146,147]. Genome analysis of bifidobacterial species has revealed that the genus *B. bifidum* have adapted to ecological niches where there is a limited source of other nutrients except carbohydrates [148]. The *B. bifidum* pan-genome consisted of 2970 COGs and the core-genome was represented by 1295 genes. Those genes were dedicated to housekeeping functions of bacterial cells such as DNA replication, transcription and translation transport and metabolism of carbohydrates and amino acids, as well as host-interacting components of bacteria including sortase-dependent pili, tight adherence (*tad*) locus and murein lytic enzyme (*TgaA*). The pili structures reported to be crucial for interacting with host and other gut microbiota [141].

Duranti et al. [141] analyzed the average abundance of bifidobacterial DNA from 11 metagenomic datasets of the gut microbiome from infants and found that the relative abundance of *B. bifidum* DNA was 12.42% in breast milk-fed infants compared with the 0.24% in formula milk-fed infants. This result was consistent with the genomic analysis representing specialized catabolic ability of *B. bifidum* to utilize host glycans in an aspect of adapting to infant gut. Turroni et al. [146] also showed the specific ability of *B. bifidum* PRL2010 metabolizing host-derived glycans, especially HMOs and mucin. A comparative genomics study based on 15 genomes of *B. bifidum* strains helped to elucidate the evolutionary force for successful adaptation of this species to specific ecological niches (i.e., infant gut) by assessing genomic variability and complexity [141,142,147]. The genetic variability of *B. bifidum* was 13.7% of the total genomic pool of *B. bifidum*, and this was relatively lower than 15.3% of mobilome value in the genus *Bifidobacterium* [141,149].

Table 3. Summary of genome analysis comparing COGs of three *B. bifidum* strains (analyzed by the authors based on NCBI datasets).

COG	Description	*B. bifidum* BGN4		*B. bifidum* PRL2010		*B. bifidum* S17	
		Number of Genes	%	Number of Genes	%	Number of Genes	%
J	Translation, ribosomal structure and biogenesis	136	10.56%	135	10.39%	135	10.48%
K	Transcription	95	7.38%	95	7.31%	93	7.22%
L	Replication, recombination and repair	102	7.92%	107	8.24%	100	7.76%
D	Cell cycle control, cell division, chromosome partitioning	24	1.86%	22	1.69%	23	1.79%
O	Posttranslational modification, protein turnover, chaperones	50	3.88%	50	3.85%	50	3.88%
M	Cell wall/membrane/envelope biogenesis	75	5.82%	81	6.24%	79	6.13%
N	Cell motility	6	0.47%	6	0.46%	5	0.39%
P	Inorganic ion transport and metabolism	50	3.88%	49	3.77%	49	3.80%
T	Signal transduction mechanisms	47	3.65%	50	3.85%	47	3.65%
C	Energy production and conversion	50	3.88%	50	3.85%	51	3.96%
G	Carbohydrate transport and metabolism	118	9.16%	117	9.01%	118	9.16%
E	Amino acid transport and metabolism	135	10.48%	137	10.55%	136	10.56%
F	Nucleotide transport and metabolism	56	4.35%	55	4.23%	56	4.35%
H	Coenzyme transport and metabolism	45	3.49%	44	3.39%	44	3.42%
I	Lipid transport and metabolism	35	2.72%	36	2.77%	36	2.80%
Q	Secondary metabolites biosynthesis, transport and catabolism	6	0.47%	7	0.54%	6	0.47%
R	General function prediction only	150	11.65%	148	11.39%	153	11.88%
S	Function unknown	108	8.39%	110	8.47%	107	8.31%
	Total	1288	100%	1299	100%	1288	100%

In case of glycobiomes, *B. bifidum* showed a relatively small size of genes and especially reduced capabilities to catabolize high-molecular plant polysaccharides. However, it should be noted that *B. bifidum* contain enriched gene-sets undertaking the metabolism of host-derived glycans and health-beneficial glyco-conjugated phytochemicals. The comparative genomic analysis of BGN4 strain with other *B. bifidum* strains and whole bifidobacterial taxa has not been reported yet. Further study focusing on identification of unique BGN4 genes, which are capable of encoding specific colonizing factors, key enzymes catalyzing HMO and glycones, and immunomodulatory molecules expressed and secreted by BGN4, should be helpful to reinforce the multiple functionality of BGN4.

9. Conclusions

This systematic review summarizes bio-functionality of BGN4 assessed by in vitro, in vivo and clinical studies and potential of BGN4 for industrial applications and explains what is known and unknown based on available data (Figure 2). To demonstrate a precise mechanism of exploitation of BGN4 for human body, multifactorial clinical research and well-controlled molecular level work should be further pursued. However, potential functional value of BGN4 was clearly established through multiple in vitro and in vivo and clinical experiments. Moreover, BGN4 has been applied practically in commercial products with a mass production. Summarized information on BGN4 would be valuable to guide design with insight of future experiments to know mechanisms of functionality, clinical trials and commercial applications.

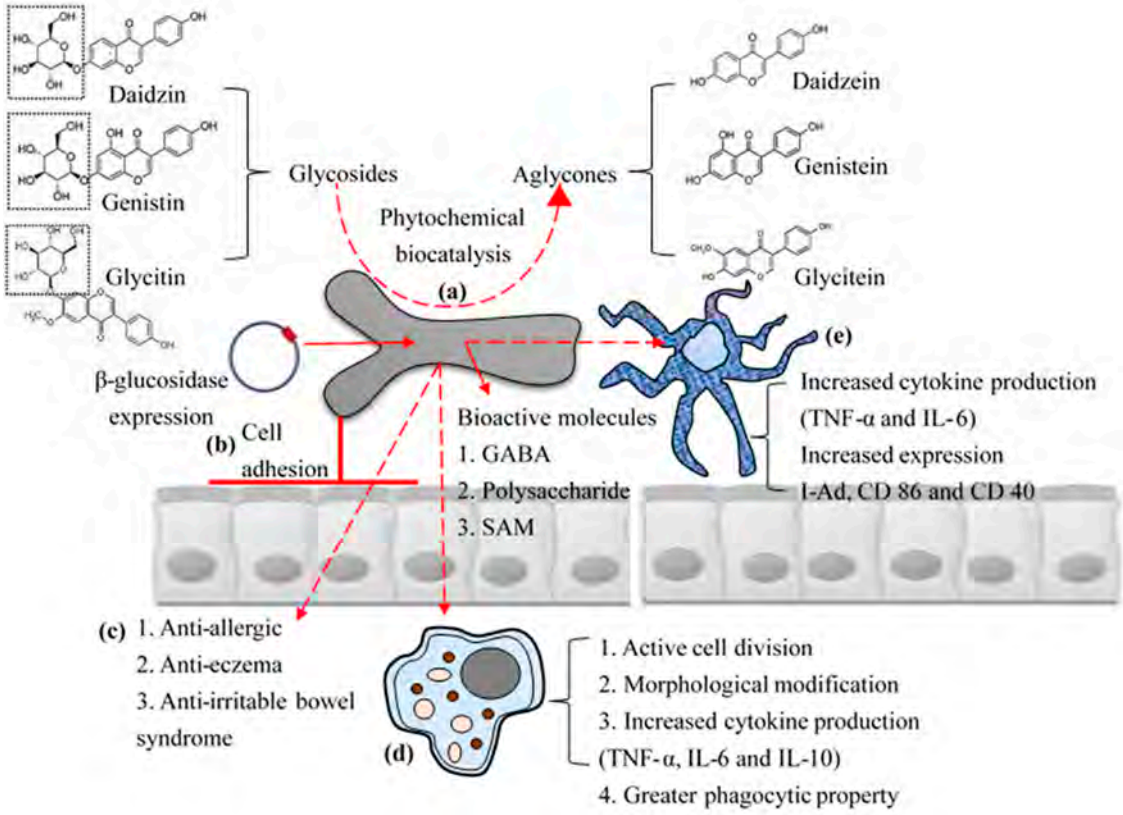

Figure 2. Schematic representation of biofunctional properties: Biotransformation of phytochemicals (**a**); high cell adhesion property with high surface hydrophobicity (**b**); and direct (**c**); and indirect immunomodulatory effects (activation of macrophages (**d**); and dendritic cells (**e**)) of *B. bifidum* BGN4 to host.

Acknowledgments: This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01123001 and PJ01123002)", Rural Development Administration of "the Promoting Regional specialized Industry (Project No. R0004140)", the Ministry of Trade,

Industry and Energy (MOTIE) and Korea Institute for Advancement of Technology (KIAT), and "Research program of SGER (Project No. NRF-2015R1D1A1A02062267)", National Research Foundation of Korea. The authors wish to thank Raymond RedCorn and Emily R. Coleman at Purdue University, and Jaycey Hardenstein of Eli Lily for their review and feedback of this paper. The authors would also like to thank Michael R. Ladisch and Eduardo Ximenes at Purdue University for their supports to Seockmo Ku.

Author Contributions: Seockmo Ku initiated this work in partial fulfillment of his degree at Seoul National University under the supervision of Geun Eog Ji, and the mentorship of Hyun Ju You and Myeong Soo Park. Seockmo Ku performed the literature search and was the primary author of the review. Seockmo Ku wrote Sections 1–7, and Section 9. Hyun Ju You wrote Section 8. Myeong Soo Park and Geun Eog Ji edited and revised the review. Geun Eog Ji and Hyun Ju You designed the review template. All authors discussed drafts and approved the final manuscript for publication.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

BGN4	*Bifidobacterium bifidum* BGN4
CD	cluster of differentiation:
FDA	USA Food and Drug Administration
GABA	γ-Aminobutyric acid
IFN	Interferon
IL	Interleukin
SAM	*S*-Adenosyl-L-Methionine
SEM	scanning electron microscope
TNF	tumor necrosis factor

References

1. Siró, I.; Kápolna, E.; Kápolna, B.; Lugasi, A. Functional food. Product development, marketing and consumer acceptance: A review. *Appetite* **2008**, *51*, 456–467. [CrossRef] [PubMed]
2. Syngai, G.; Gopi, R.; Bharali, R.; Dey, S.; Lakshmanan, G.; Ahmed, G. Probiotics—The versatile vunctional food ingredients. *J. Food Sci. Technol.* **2015**, *53*, 921–933. [CrossRef] [PubMed]
3. U.S. Food and Drug Administration, Labeling & Nutrition. Available online: http://www.fda.gov/Food/IngredientsPackagingLabeling/LabelingNutrition/ (accessed on 30 June 2016).
4. De Prisco, A.; Mauriello, G. Probiotication of foods: A focus on microencapsulation tool. *Trends Food Sci. Technol.* **2016**, *48*, 27–39. [CrossRef]
5. Yildiz, F. *Development and Manufacture of Yogurt and Other Functional Dairy Products*; CRC Press/Taylor & Francis: Boca Raton, FL, USA, 2010; pp. 1–36.
6. Preedy, V. *Handbook of Diet, Nutrition and The Skin*; Wageningen Academic Publisher: Wageningen, The Netherland, 2012; pp. 327–328.
7. Sarkar, S.; Sur, A.; Sarkar, K.; Majhi, R.; Basu, S.; Chatterjee, K.; Sikder, B. Probiotics: A way of value addition in functional food. *Int. J. Food Sci. Nutr. Diet.* **2016**, *5*, 290–293.
8. Tajabadi, N.; Ebrahimpour, A.; Baradaran, A.; Rahim, R.; Mahyudin, N.; Manap, M.; Bakar, F.; Saari, N. Optimization Of γ-Aminobutyric acid production by *Lactobacillus plantarum* Taj-Apis362 from honeybees. *Molecules* **2015**, *20*, 6654–6669. [CrossRef] [PubMed]
9. Ku, S. Finding and producing probiotic glycosylases for the biocatalysis of ginsenosides: A mini review. *Molecules* **2016**, *21*, 645. [CrossRef] [PubMed]
10. Soto, A.; Martín, V.; Jiménez, E.; Mader, I.; Rodríguez, J.; Fernández, L. Lactobacilli and bifidobacteria in human bBreast milk: Influence of antibiotherapy and other host and clinical factors. *J. Pediatr. Gastroenterol. Nutr.* **2014**, *59*, 78–88. [CrossRef] [PubMed]
11. Arboleya, S.; Ruas-Madiedo, P.; Margolles, A.; Solís, G.; Salminen, S.; Clara, G.; Gueimonde, M. Characterization and in vitro properties of potentially probiotic bifidobacterium strains isolated from breast-milk. *Int. J. Food Microbiol.* **2011**, *149*, 28–36. [CrossRef] [PubMed]
12. O'Sullivan, A.; Farver, M.; Smilowitz, J. The influence of early infant-feeding practices on the intestinal microbiome and body composition in infants. *Nutr. Metab. Insights* **2015**, *8* (Suppl. S1), 1–9. [PubMed]
13. Grguric, J.; Percl, M.; Kolacek, S.; Bacic, V. Microflora in the digestive tract of infants. *Mljekarstvo* **1996**, *46*, 291–296.

14. Saavedra, J.M. Use of probiotics in pediatrics: Rationale, mechanisms of action, and practical aspects. *Nutr. Clin. Pract.* **2007**, *22*, 351–365. [CrossRef] [PubMed]
15. Lewis, Z.T.; Totten, S.M.; Smilowitz, J.T.; Popovic, M.; Parker, E.; Lemay, D.G.; van Tassell, M.L.; Miller, M.J.; Jin, Y.S.; German, J.B.; et al. Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. *Microbiome* **2015**, *3*. [CrossRef] [PubMed]
16. Harmsen, H.J.; Wildeboer–Veloo, A.C.; Raangs, G.C.; Wagendorp, A.A.; Klijn, N.; Bindels, J.G.; Welling, G.W. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J. Pediatr. Gastroenterol. Nutr.* **2000**, *30*, 61–67. [CrossRef] [PubMed]
17. Aggett, P.J.; Agostoni, C.; Axelsson, I.; Edwards, C.A.; Goulet, O.; Hernell, O.; Koletzko, B.; Lafeber, H.N.; Micheli, J.L.; Michaelsen, K.F.; et al. Nondigestible carbohydrates in the diets of infants and young children: A commentary by the ESPGHAN Committee on Nutrition. *J. Pediatr. Gastroenterol. Nutr.* **2003**, *36*, 329–337. [CrossRef] [PubMed]
18. Turroni, F.; Peano, C.; Pass, D.; Foroni, E.; Severgnini, M.; Claesson, M.; Kerr, C.; Hourihane, J.; Murray, D.; Fuligni, F.; et al. Diversity of bifidobacteria within the infant gut microbiota. *PLoS ONE* **2012**, *7*, e36957. [CrossRef] [PubMed]
19. Turroni, F.; Duranti, S.; Bottacini, F.; Guglielmetti, S.; van Sinderen, D.; Ventura, M. *Bifidobacterium bifidum* as an example of a specialized human gut commensal. *Front. Microbiol.* **2014**, *5*. [CrossRef] [PubMed]
20. Guglielmetti, S.; Mora, D.; Gschwender, M.; Popp, K. Randomised clinical trial: *Bifidobacterium bifidum* MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life—A double-BLIND, Placebo-Controlled Study. *Aliment. Pharmacol. Ther.* **2011**, *33*, 1123–1132. [CrossRef] [PubMed]
21. Wang, Y.; Huang, Y. Effect of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* supplementation to standard triple therapy on Helicobacter pylori eradication and dynamic changes in intestinal flora. *World J. Microbiol. Biotechnol.* **2013**, *30*, 847–853. [CrossRef] [PubMed]
22. Toiviainen, A.; Jalasvuori, H.; Lahti, E.; Gursoy, U.; Salminen, S.; Fontana, M.; Flannagan, S.; Eckert, G.; Kokaras, A.; Paster, B.; et al. Impact of orally administered lozenges with *lactobacillus rhamnosus* GG and *bifidobacterium animalis* subsp. Lactis BB-12 on the number of salivary mutans streptococci, amount of plaque, gingival inflammation and the oral microbiome in healthy adults. *Clin. Oral. Investig.* **2014**, *19*, 77–83. [CrossRef] [PubMed]
23. Culpepper, T.; Christman, M.; Nieves, C.; Specht, G.; Rowe, C.; Spaiser, S.; Ford, A.; Dahl, W.; Girard, S.; Langkamp-Henken, B. *Bifidobacterium bifidum* R0071 decreases stress-associated diarrhoea-related symptoms and self-reported stress: A secondary analysis of a randomised trial. *Benef. Microbes* **2016**, *7*, 327–336. [CrossRef] [PubMed]
24. Choi, Y.J.; Kim, C.J.; Park, S.Y.; Ko, Y.T.; Jeong, H.K.; Ji, G.E. Growth and β-Glucosidase activity of bifidobacterium. *J. Microbiol. Biotechnol.* **1996**, *6*, 255–259.
25. BIFIDO. Available online: http://www.bifido.com/en/product/probiotics/health?category=zigunuk (accessed on 30 June 2016).
26. Kim, I.H.; Park, M.S.; Ji, G.E. Characterization of adhesion of *Bifidobacterium* sp. BGN4 to human enterocyte-like Caco-2 cells. *J. Microbiol. Biotechnol.* **2003**, *13*, 276–281.
27. Ku, S.; You, H.J.; Ji, G.E. Enhancement of anti-tumorigenic polysaccharide production, adhesion, and branch formation of Bifidobacterium bifidum BGN4 by phytic acid. *Food Sci. Biotechnol.* **2009**, *18*, 749–754.
28. Lee, M.J.; Zang, Z.; Choi, E.Y.; Shin, H.K.; Ji, G.E. Cytoskeleton reorganization and cytokine production of macrophages by bifidobacterial cells and cell-free extracts. *J. Microbiol. Biotechnol.* **2002**, *12*, 398–405.
29. Kim, N.; Ji, G.E. Modulatory activity of *Bifidobacterium* sp. BGN4 cell fractions on immune cells. *J. Microbiol. Biotechnol.* **2006**, *16*, 584–589.
30. Lee, S.; Koo, N.; Oh, S. Regulatory effect on specific ige response of *Bifidobacterium bifidum* (BGN4 Strain) in murine model of peanut allergy. *J. Allergy Clin. Immunol.* **2006**, *117*, S204. [CrossRef]
31. Kim, N.; Kunisawa, J.; Kweon, M.; Ji, G.E.; Kiyono, H. Oral feeding of *Bifidobacterium bifidum* (BGN4) Prevents CD4 + CD45RB high T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. *Clin. Immunol.* **2007**, *123*, 30–39. [CrossRef] [PubMed]
32. Hong, K.S.; Kang, H.W.; Im, J.P.; Ji, G.E.; Kim, S.G.; Jung, H.C.; Song, I.S.; Kim, J.S. Effect of probiotics on symptoms in Korean adults with irritable bowel syndrome. *Gut Liver* **2009**, *3*, 101–107. [CrossRef] [PubMed]

33. Kim, J.Y.; Kwon, J.H.; Ahn, S.H.; Lee, S.I.; Han, Y.S.; Choi, Y.O.; Lee, S.Y.; Ahn, K.M.; Ji, G.E. Effect of probiotic mix (*Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*) in the primary prevention of eczema: A double-blind, randomized, placebo-controlled trial. *Pediatr. Allergy Immunol.* **2010**, *21*, 386–393. [CrossRef] [PubMed]
34. Kim, J.Y.; Park, M.S.; Ji, G.E. Probiotic modulation of dendritic cells co-cultured with intestinal epithelial cells. *World J. Gastroenterol.* **2012**, *18*, 1308–1318. [CrossRef] [PubMed]
35. Shim, J.Y.; Kim, Y.K.; Ji, G.E.; Om, A.S. Effect of fermented soymilk using *Bifidobacterium* spp. RD65 and BGN4 on abberant crypt foci in azoxymethane induced colon cancer rats. *KoSFoST Int. Symp. Annu. Meet.* **2001**, 197.
36. You, H.J.; Oh, D.K.; Ji, G.E. Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from *Bifidobacterium bifidum* BGN4. *FEMS Microbiol. Lett.* **2004**, *240*, 131–136. [CrossRef] [PubMed]
37. Youn, S.Y.; Park, M.S.; Ji, G.E. Identification of the β-glucosidase gene from *Bifidobacterium animalis* Subsp. *Lactis* and its expression in *B. bifidum* BGN4. *J. Microbiol. Biotechnol.* **2012**, *22*, 1714–1723.
38. Kim, J.Y.; Wang, Y.; Park, S.J.; Ji, G.E.; Park, M.S. Cloning and expression of β-Glucosidases from *Bifidobacterium lactis* AD011. *Food Sci. Biotechnol.* **2012**, *21*, 731–738. [CrossRef]
39. Wang, Y.; Kim, J.Y.; Park, M.S.; Ji, G.E. Novel *Bifidobacterium* promoters selected through microarray analysis lead to constitutive high-level gene expression. *J. Microbiol.* **2012**, *50*, 638–643. [CrossRef] [PubMed]
40. Kim, J.A.; Park, M.S.; Kang, S.A.; Ji, G.E. Production of γ-aminobutyric acid during fermentation of *Gastrodia Elata* Bl. By co-culture of *Lactobacillus brevis* GABA 100 with *Bifidobacterium bifidum* BGN4. *Food Sci. Biotechnol.* **2014**, *23*, 459–466. [CrossRef]
41. You, H.J.; Ahn, H.J.; Kim, J.Y.; Wu, Q.Q.; Ji, G.E. High expression of β-glucosidase in *Bifidobacterium bifidum* BGN4 and application in conversion of isoflavone glucosides during fermentation of soy milk. *J. Microbiol. Biotechnol.* **2015**, *25*, 469–478. [CrossRef] [PubMed]
42. Kim, J.Y.; Seo, H.S.; Seo, J.M.; Suh, J.W.; Hwang, I.; Ji, G.E. Development of *S*-adenosyl-L-methionine (SAM)-reinforced probiotic yogurt using *Bifidobacterium bifidum* BGN4. *Food Sci. Biotechnol.* **2008**, *17*, 1025–1031.
43. Kim, J.Y.; Suh, J.W.; Ji, G.E. Evaluation of *S*-adenosyl-L-methionine production by *Bifidobacterium bifidum* BGN4. *Food Sci. Biotechnol.* **2008**, *17*, 184–187.
44. Yu, D.S.; Jeong, H.; Lee, D.H.; Kwon, S.K.; Song, J.Y.; Kim, B.Y.; Park, M.S.; Ji, G.E.; Oh, T.K.; Kim, J.F. Complete genome sequence of the probiotic bacterium *Bifidobacterium bifidum* strain BGN4. *J. Bacteriol.* **2012**, *194*, 4757–4758. [CrossRef] [PubMed]
45. U.S. Food and Drug Administration, Bad Bug Book (Second Edition). Available online: http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/ (accessed on 30 June 2016).
46. Ribet, D.; Cossart, P. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes Infect.* **2015**, *17*, 173–183. [CrossRef] [PubMed]
47. Thöle, C.; Brandt, S.; Ahmed, N.; Hensel, A. Acetylated rhamnogalacturonans from immature fruits of Abelmoschus esculentus inhibit the adhesion of Helicobacter pylori to human gastric cells by interaction with outer membrane proteins. *Molecules* **2015**, *20*, 16770–16787. [CrossRef] [PubMed]
48. Kim, J.K.; Shin, E.C.; Park, H.G. Fructooligosaccharides decreased the ability of probiotic Escherichia coli Nissle 1917 to adhere to co-cultures of human intestinal cell lines. *J. Korean Soc. Appl. Biol. Chem.* **2015**, *58*, 45–52. [CrossRef]
49. Serafini, F.; Strati, F.; Ruas-Madiedo, P.; Turroni, F.; Foroni, E.; Duranti, S.; Milano, F.; Perotti, A.; Viappiani, A.; Guglielmetti, S.; et al. Evaluation of adhesion properties and antibacterial activities of the infant gut commensal *Bifidobacterium bifidum* PRL2010. *Anaerobe* **2013**, *21*, 9–17. [CrossRef] [PubMed]
50. Underwood, M.; Kananurak, A.; Coursodon, C.; Adkins-Reick, C.; Chu, H.; Bennett, S.; Wehkamp, J.; Castillo, P.; Leonard, B.; Tancredi, D.; et al. *Bifidobacterium bifidum* in a rat model of necrotizing enterocolitis: Antimicrobial peptide and protein responses. *Pediatr. Res.* **2012**, *71*, 546–551. [CrossRef] [PubMed]
51. Martinez, F.A.; Balciunas, E.M.; Converti, A.; Cotter, P.D.; de Souza Oliveira, R.P. Bacteriocin production by *Bifidobacterium* spp: A review. *Biotechnol. Adv.* **2013**, *31*, 482–488. [CrossRef] [PubMed]
52. Mandal, S.M.; Silva, O.N.; Franco, O.L. Recombinant probiotics with antimicrobial peptides: A dual strategy to improve immune response in immunocompromised patients. *Drug Discov. Today* **2014**, *19*, 1045–1050. [CrossRef] [PubMed]

53. Slavin, J. Fiber and prebiotics: Mechanisms and health benefits. *Nutrients* **2013**, *5*, 1417–1435. [CrossRef] [PubMed]
54. Aronsson, L.; Huang, Y.; Parini, P.; Korach-André, M.; Håkansson, J.; Gustafsson, J.; Pettersson, S.; Arulampalam, V.; Rafter, J. Decreased fat storage by *Lactobacillus paracasei* is associated with increased levels of angiopoietin-like 4 protein (ANGPTL4). *PLoS ONE* **2010**, *5*, e13087. [CrossRef] [PubMed]
55. DiBaise, J.K.; Frank, D.N.; Mathur, R. Impact of the gut microbiota on the development of obesity: Current concepts. *Am. J. Gastroenterol. Suppl.* **2012**, *1*, 22–27. [CrossRef]
56. Hardy, H.; Harris, J.; Lyon, E.; Beal, J.; Foey, A. Probiotics, prebiotics and immunomodulation of gut mucosal defences: Homeostasis and immunopathology. *Nutrients* **2013**, *5*, 1869–1912. [CrossRef] [PubMed]
57. Yu, Q.; Wang, Z.; Yang, Q. Ability of *Lactobacillus* to inhibit enteric pathogenic bacteria adhesion on Caco-2 cells. *World J. Microbiol. Biotechnol.* **2010**, *27*, 881–886. [CrossRef]
58. Kim, B.J.; Hong, J.H.; Jeong, Y.S.; Jung, H.K. Evaluation of two Bacillus subtilis strains isolated from Korean fermented food as probiotics against loperamide-induced constipation in mice. *J. Korean Soc. Appl. Biol. Chem.* **2014**, *57*, 797–806. [CrossRef]
59. Lim, S.M. Anti-helicobacter pylori activity of antimicrobial substances produced by lactic acid bacteria isolated from Baikkimchi. *J. Korean Soc. Appl. Biol. Chem.* **2014**, *57*, 621–630. [CrossRef]
60. Lebeer, S.; Vanderleyden, J.; De Keersmaecker, S.C. Host interactions of probiotic bacterial surface molecules: Comparison with commensals and pathogens. *Nat. Rev. Microbiol.* **2010**, *8*, 171–184. [CrossRef] [PubMed]
61. Van Tassell, M.; Miller, M. *Lactobacillus* adhesion to mucus. *Nutrients* **2011**, *3*, 613–636. [PubMed]
62. Duary, R.K.; Rajput, Y.S.; Batish, V.K.; Grover, S. Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J. Med. Res.* **2011**, *134*, 664–671. [PubMed]
63. Polak-Berecka, M.; Waśko, A.; Paduch, R.; Skrzypek, T.; Sroka-Bartnicka, A. The effect of cell surface components on adhesion ability of *Lactobacillus* rhamnosus. *Antonie van Leeuwenhoek* **2014**, *106*, 751–762. [CrossRef] [PubMed]
64. Krasowska, A.; Sigler, K. How microorganisms use hydrophobicity and what does this mean for human needs? *Front. Cell. Infect. Microbiol.* **2014**, *4*, 112. [CrossRef] [PubMed]
65. Pelletier, C.; Bouley, C.; Cayuela, C.; Bouttier, S.; Bourlioux, P.; Bellon-Fontaine, M. Cell surface characteristics of *Lactobacillus casei* subsp. *casei*, *Lactobacillus paracasei* subsp. *paracasei*, and *Lactobacillus rhamnosus* strains. *Appl. Environ. Microbiol.* **1997**, *63*, 1725–1731. [PubMed]
66. Abdulla, A.A.; Abed, T.A.; Saeed, A.M. Adhesion, Autoaggregation and hydrophobicity of six *Lactobacillus* strains. *Br. Microbiol. Res. J.* **2014**, *4*, 381–391. [CrossRef]
67. Boris, S.; Suárez, J.E.; Vázquez, F.; Barbés, C. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infect. Immun.* **1998**, *66*, 1985–1989. [PubMed]
68. Shakirova, L.; Auzina, L.; Zikmanis, P.; Gavare, M.; Grube, M. Influence of growth conditions on hydrophobicity of *Lactobacillus acidophilus* and *Bifidobacterium lactis* cells and characteristics by FT-IR spectra. *J. Spectrosc.* **2010**, *24*, 251–255. [CrossRef]
69. Pérez, P.F.; Minnaard, Y.; Disalvo, E.A.; de Antoni, G.L. Surface properties of bifidobacterial strains of human origin. *J. Microbiol. Biotechnol.* **1998**, *64*, 21–26.
70. Pan, W.; Li, P.; Liu, Z. The correlation between surface hydrophobicity and adherence of *Bifidobacterium* strains from centenarians' faeces. *Anaerobe* **2006**, *12*, 148–152. [CrossRef] [PubMed]
71. Schillinger, U.; Guigas, C.; Holzapfel, W.H. In vitro adherence and other properties of lactobacilliused in probiotic yoghurt-like products. *Int. Dairy J.* **2005**, *15*, 1289–1297. [CrossRef]
72. Alzate, A.; Fernandez, A.; Perez-Conde, M.C.; Gutierrez, A.M.; Camara, C. Comparison of biotransformation of inorganic selenium by *Lactobacillus* and *Saccharomyces* in lactic fermentation process of yogurt and kefir. *J. Agric. Food Chem.* **2008**, *56*, 8728–8736. [CrossRef] [PubMed]
73. Botes, M.; Loos, B.; van Reenen, C.A.; Dicks, L.M.T. Adhesion of the probiotic strains *enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments. *Arch. Microbiol.* **2008**, *190*, 573–584. [CrossRef] [PubMed]
74. Wadström, T.; Andersson, K.; Sydow, M.; Axelsson, L.; Lindgren, S.; Gullmar, B. Surface properties of lactobacilli isolated from the small intestine of pigs. *J. Appl. Bacteriol.* **1987**, *62*, 513–520. [CrossRef] [PubMed]

75. Candela, M.; Perna, F.; Carnevali, P.; Vitali, B.; Ciati, R.; Gionchetti, P.; Rizzello, F.; Campieri, M.; Brigidi, P. Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: Adhesion properties, competition against enteropathogens and modulation of IL-8 production. *Int. J. Food Microbiol.* **2008**, *125*, 286–292. [CrossRef] [PubMed]
76. Crociani, J.; Grill, J.P.; Huppert, M.; Ballongue, J. Adhesion of different bifidobacteria strains to human enterocyte-like Caco-2 cells and comparison with in vivo study. *Lett. Appl. Microbiol.* **1995**, *21*, 146–148. [CrossRef] [PubMed]
77. Borchers, A.T.; Selmi, C.; Meyers, F.J.; Keen, C.L.; Gershwin, M.E. Probiotics and immunity. *J. Gastroenterol.* **2009**, *44*, 26–46. [CrossRef] [PubMed]
78. Galdeano, C.M.; de LeBlanc, A.D.; Vinderola, G.; Bonet, M.B.; Perdigon, G. Proposed model: Mechanisms of immunomodulation induced by probiotic bacteria. *Clin. Vaccine Immunol.* **2007**, *14*, 485–492. [CrossRef] [PubMed]
79. Martinez, F.; Gordon, S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Rep.* **2014**, *6*. [CrossRef] [PubMed]
80. Kim, B.I.; Joo, Y.H.; Pak, P.J.; Kim, J.S.; Chung, N. Different shapes of Al2O3 particles induce differential cytotoxicity via a mechanism involving lysosomal destabilization and reactive oxygen species generation. *J. Korean Soc. Appl. Biol. Chem.* **2015**, *58*, 433–442. [CrossRef]
81. Li, X.; Bao, W.; Leung, C.; Ma, D.; Zhang, G.; Lu, A.; Wang, S.; Han, Q. Chemical structure and immunomodulating activities of an α-glucan purified from *Lobelia chinensis* lour. *Molecules* **2016**, *21*, 779. [CrossRef] [PubMed]
82. Cui, S.; Hassan, R.; Heintz-Buschart, A.; Bilitewski, U. Regulation of *Candida albicans* interaction with macrophages through the activation of HOG pathway by genistein. *Molecules* **2016**, *21*, 162. [CrossRef] [PubMed]
83. Gill, H.S.; Rutherfurd, K.J.; Cross, M.L.; Gopal, P.K. Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. *Am. J. Clin. Nutr.* **2001**, *74*, 833–839. [PubMed]
84. Fong, F.L.; Shah, N.P.; Kirjavainen, P.; El-Nezami, H. Mechanism of action of probiotic bacteria on intestinal and systemic immunities and antigen-presenting cells. *Int. Rev. Immunol.* **2015**, 1–11. [CrossRef] [PubMed]
85. World Cancer Research Fund International, Colorectal Cancer Statistics. Available online: http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/colorectal-cancer-statistics (accessed on 5 July 2016).
86. American Cancer Society, Key Statistics for Colorectal Cancer. Available online: http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-key-statistics (accessed on 12 July 2016).
87. Pourhoseingholi, M. Increased burden of colorectal cancer in Asia. *World J. Gastrointest. Oncol.* **2012**, *4*, 68–70. [CrossRef] [PubMed]
88. Selhub, E.; Logan, A.; Bested, A. Fermented foods, microbiota, and mental health: Ancient practice meets nutritional psychiatry. *J. Physiol. Anthropol.* **2014**, *33*. [CrossRef] [PubMed]
89. Kumar, M.; Nagpal, R.; Verma, V.; Kumar, A.; Kaur, N.; Hemalatha, R.; Gautam, S.; Singh, B. Probiotic metabolites as epigenetic targets in the prevention of colon cancer. *Nutr. Rev.* **2012**, *71*, 23–34. [CrossRef] [PubMed]
90. Choi, S.S.; Kim, Y.; Han, K.S.; You, S.; Oh, S.; Kim, S.H. Effects of *Lactobacillus* strains on cancer cell proliferation and oxidative stress in vitro. *Lett. Appl. Microbiol.* **2006**, *42*, 452–458. [CrossRef] [PubMed]
91. Uccello, M.; Malaguarnera, G.; Basile, F.; D'agata, V.; Malaguarnera, M.; Bertino, G.; Vacante, M.; Drago, F.; Biondi, A. Potential role of probiotics on colorectal cancer prevention. *BMC Surg.* **2012**, *12*, S35. [CrossRef] [PubMed]
92. Raman, M.; Ambalam, P.; Doble, M. *Probiotics and Bioactive Carbohydrates in Colon Cancer Management*; Springer (India) Pvt. Ltd.: New Delhi, India, 2016; pp. 83–109.
93. Sadeghi-Aliabadi, H.; Mohammadi, F.; Fazeli, H.; Mirlohi, M. Effects of *Lactobacillus plantarum* A7 with probiotic potential on colon cancer and normal cells proliferation in comparison with a commercial strain. *Iran J. Basic Med. Sci.* **2014**, *17*, 815–819. [PubMed]
94. Nagaoka, M.; Hashimoto, S.; Watanabe, T.; Yokokura, T.; Mori, Y. Anti-ulcer effects of lactic acid bacteria and their cell wall polysaccharides. *Biol. Pharm. Bull.* **1994**, *17*, 1012–1017. [CrossRef] [PubMed]
95. Van Breemen, R.; Li, Y. Caco-2 cell permeability assays to measure drug absorption. *Expert Opin. Drug. Metab. Toxicol.* **2005**, *1*, 175–185. [CrossRef] [PubMed]

96. Corpet, D.; Tache, S. Most effective colon cancer chemopreventive agents in rats: A systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr. Cancer* **2002**, *43*, 1–21. [CrossRef] [PubMed]
97. Kim, J.; Ng, J.; Arozulllah, A.; Ewing, R.; Llor, X.; Carroll, R.E.; Benya, R.V. Aberrant crypt focus size predicts distal polyp histopathology. *Cancer Epidemiol. Biomark. Prev.* **2008**, *17*, 1155–1162. [CrossRef] [PubMed]
98. Foerst, P.; Santivarangkna, C. *Advances in Probiotic Technology*; CRC Press/Taylor & Francis: Boca Raton, FL, USA, 2015; pp. 356–374.
99. Foligné, B.; Daniel, C.; Pot, B. Probiotics from research to market: The possibilities, risks and challenges. *Curr. Opin. Microbiol.* **2013**, *16*, 284–292. [CrossRef] [PubMed]
100. Lahtinen, S. Probiotic viability—Does it matter? *Microb. Ecol. Health Dis.* **2012**, *23*, 10. [CrossRef] [PubMed]
101. Nedović, V.; Raspor, P.; Lević, J.; Tumbas Šaponjac, V.; Barbosa-Cánovas, G. *Emerging and Traditional Technologies for Safe, Healthy and Quality Food*; Springer International Publishing: Cham, Germany, 2016; pp. 257–268.
102. Park, S.J.; Youn, S.Y.; Ji, G.E.; Park, M.S. Whole cell biotransformation of major ginsenosides using leuconostocs and lactobacilli. *Food Sci. Biotechnol.* **2012**, *21*, 839–844. [CrossRef]
103. You, H.J.; Ahn, H.J.; Ji, G.E. Transformation of Rutin to antiproliferative quercetin-3-glucoside by *Aspergillus niger*. *J. Agric. Food Chem.* **2010**, *58*, 10886–10892. [CrossRef] [PubMed]
104. Ku, S.; Zheng, H.; Park, M.S.; Ji, G.E. Optimization of β-glucuronidase activity from *Lactobacillus delbrueckii* Rh2 and its use for biotransformation of baicalin and wogonoside. *J. Korean Soc. Appl. Biol. Chem.* **2011**, *54*, 275–280. [CrossRef]
105. Ku, S.; You, H.J.; Park, M.S.; Ji, G.E. Effects of ascorbic acid on α-L-arabinofuranosidase and α-L-arabinopyranosidase activities from *Bifidobacterium longum* RD47 and its application to whole cell bioconversion of ginsenoside. *J. Korean Soc. Appl. Biol. Chem.* **2015**, *58*, 857–865. [CrossRef] [PubMed]
106. Ku, S.; You, H.J.; Park, M.S.; Ji, G.E. Whole-cell biocatalysis for producing ginsenoside Rd from Rb1 using *Lactobacillus rhamnosus* GG. *J. Microbiol. Biotechnol.* **2016**, *26*, 1206–1215. [CrossRef] [PubMed]
107. Gao, F.; Zhang, J.M.; Wang, Z.G.; Peng, W.; Hu, H.L.; Fu, C.M. Biotransformation, a promising technology for anti-cancer drug development. *Asian Pac. J. Cancer Prev.* **2013**, *14*, 5599–5608. [CrossRef] [PubMed]
108. Sasidharan, S.; Chen, Y.; Saravanan, D.; Sundram, K.; Latha, L. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *Afr. J. Tradit. Complement Altern. Med.* **2011**, *8*, 1–10. [CrossRef] [PubMed]
109. Han, B.H.; Park, M.H.; Han, Y.N.; Woo, L.K.; Sankawa, U.; Yahara, S.; Tanaka, O. Degradation of ginseng saponins under mild acidic conditions. *Planta Med.* **1982**, *44*, 146–149. [CrossRef] [PubMed]
110. Chen, Y.; Nose, M.; Ogihara, Y. Alkaline cleavage of ginsenosides. *Chem. Pharm. Bull.* **1987**, *35*, 1653–1655. [CrossRef] [PubMed]
111. Nag, S.A.; Qin, J.J.; Wang, W.; Wang, M.H.; Wang, H.; Zhang, R. Ginsenosides as anticancer agents: In vitro and in vivo activities, structure-activity relationships, and molecular mechanisms of action. *Front. Pharmacol.* **2012**, *3*. [CrossRef] [PubMed]
112. Taku, K.; Melby, M.; Nishi, N.; Omori, T.; Kurzer, M. Soy isoflavones for osteoporosis: An evidence-based approach. *Maturitas* **2011**, *70*, 333–338. [CrossRef] [PubMed]
113. Orgaard, A.; Jensen, L. The effects of soy isoflavones on obesity. *Exp. Biol. Med.* **2008**, *233*, 1066–1080. [CrossRef] [PubMed]
114. Lu, M.; Wang, R.; Song, X.; Chibbar, R.; Wang, X.; Wu, L.; Meng, Q. Dietary soy isoflavones increase insulin secretion and prevent the development of diabetic cataracts in streptozotocin-induced diabetic rats. *Nutr. Res.* **2008**, *28*, 464–471. [CrossRef] [PubMed]
115. Rafii, F. The role of colonic bacteria in the metabolism of the natural isoflavone daidzin to equol. *Metabolites* **2015**, *5*, 56–73. [CrossRef] [PubMed]
116. Kennedy, A.R. The evidence for soybean products as cancer preventive agents. *J. Nutr.* **1995**, *125*, 733–743.
117. Bawa, S. The significance of soy protein and soy bioactive compounds in the prophylaxis and treatment of osteoporosis. *J. Osteoporos.* **2010**, *2010*, 1–8. [CrossRef] [PubMed]
118. Kuo, L.; Wu, R.; Lee, K. A process for high-efficiency isoflavone deglycosylation using *Bacillus subtilis* natto NTU-18. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1181–1188. [CrossRef] [PubMed]
119. New York Times, Stop Bashing G.M.O. Foods, More Than 100 Nobel Laureates Say. Available online: http://www.nytimes.com/2016/07/01/us/stop-bashing-gmo-foods-more-than-100-nobel-laureates-say.html?_r=0 (accessed on 12 July 2016).

120. Hayakawa, K.; Kimura, M.; Kasaha, K.; Matsumoto, K.; Sansawa, H.; Yamori, Y. Effect of a γ-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Br. J. Nutr.* **2004**, *92*, 411–417. [CrossRef] [PubMed]
121. Adeghate, E.; Ponery, A. GABA in the endocrine pancreas: Cellular localization and function in normal and diabetic rats. *Tissue Cell* **2002**, *34*, 1–6. [CrossRef] [PubMed]
122. Di Cagno, R.; Mazzacane, F.; Rizzello, C.; de Angelis, M.; Giuliani, G.; Meloni, M.; de Servi, B.; Gobbetti, M. Synthesis of γ-aminobutyric acid (GABA) by *Lactobacillus* plantarum DSM19463: Functional grape must beverage and dermatological applications. *Appl. Microbiol. Biotechnol.* **2009**, *86*, 731–741. [CrossRef] [PubMed]
123. Li, H.; Qiu, T.; Huang, G.; Cao, Y. Production of γ-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microb. Cell Fact.* **2010**, *9*. [CrossRef] [PubMed]
124. Huh, K.; Yi, S.J.; Shin, U.S.; Park, J.M. Effect of the ether fraction of *Gastrodia elata* methanol extract on the pentylenetetrazole-induced seizures. *J. Appl. Pharmacol.* **1995**, *3*, 199–204.
125. Lee, O.H.; Kim, K.I.; Han, C.K.; Kim, Y.C.; Hong, H.D. Effects of acidic polysaccharides from *Gastrodia rhizome* on systolic blood pressure and serum lipid concentrations in spontaneously hypertensive rats fed a high-fat diet. *Int. J. Mol. Sci.* **2012**, *13*, 698–709. [CrossRef] [PubMed]
126. Kim, J.Y.; Lee, M.Y.; Ji, G.E.; Lee, Y.S.; Hwang, K.T. Production of γ-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. *Int. J. Food Microbiol.* **2009**, *130*, 12–16. [CrossRef] [PubMed]
127. Komatsuzaki, N.; Shima, J.; Kawamoto, S.; Momose, H.; Kimura, T. Production of γ-aminobutyric acid (gaba) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol.* **2005**, *22*, 497–504. [CrossRef]
128. Matos, J.R.; Raushel, F.; Wong, C.H. *S*-adenosylmethionine: Studies on chemical and enzymatic synthesis. *Biotechnol. Appl. Biochem.* **1987**, *9*, 39–52. [PubMed]
129. Papakostas, G.; Alpert, J.; Fava, M. S-adenosyl-methionine in depression: A comprehensive review of the literature. *Curr. Psychiatry Rep.* **2003**, *5*, 460–466. [CrossRef] [PubMed]
130. Lieber, C.S. S-adenosyl-L-methionine: Its role in the treatment of liver disorders. *Am. J. Clin. Nutr.* **2002**, *76*, 1183–1187.
131. Häuser, W.; Bernardy, K.; Üçeyler, N.; Sommer, C. Treatment of fibromyalgia syndrome with antidepressants. *J. Gen. Intern. Med.* **2009**, *301*, 198–209. [CrossRef] [PubMed]
132. Armando, M.; Galvagno, M.; Dogi, C.; Cerrutti, P.; Dalcero, A.; Cavaglieri, L. Statistical optimization of culture conditions for biomass production of probiotic gut-borne *Saccharomyces cerevisiae* strain able to reduce fumonisin B1. *J. Appl. Microbiol.* **2013**, *114*, 1338–1346. [CrossRef] [PubMed]
133. Rani, M.; Appaiah, A. Optimization of culture conditions for bacterial cellulose production from *Gluconacetobacter hansenii* UAC09. *Ann. Microbiol.* **2011**, *61*, 781–787. [CrossRef]
134. Kwon, S.G.; Son, J.W.; Kim, H.J.; Park, C.S.; Lee, J.K.; Ji, G.E.; Oh, D.K. High concentration cultivation of *Bifidobacterium bifidum* in a submerged membrane bioreactor. *Biotechnol. Prog.* **2006**, *22*, 1591–1597. [CrossRef] [PubMed]
135. Ji, G.E.; Ku, S.; Park, M.S. Culture medium Containing Phytic Acid for Cultivation of *Bifidobacterium bifidum* BGN4 and Method for Production of *Bifidobacterium bifidum* BGN4 Polysaccharide Using the Medium. S. Korea Patent 1010377780000, 23 May 2011.
136. Dias, F.F.; Okrend, H.; Dondero, N.C. Calcium nutrition of *Sphaerotilus* growing in a continuous-flow apparatus. *Appl. Microbiol.* **1968**, *16*, 1364–1369. [PubMed]
137. Snellen, J.E.; Raj, H.D. Morphogenesis and fine structure of *Leucothrix mucor* and effects of calcium deficiency. *J. Bacteriol.* **1970**, *101*, 240–249. [PubMed]
138. Wright, C.T.; Klaenhammer, T.R. Calcium-induced alteration of cellular morphology affecting the resistance of *Lactobacillus acidophilus* to freezing. *Appl. Environ. Microbiol.* **1981**, *41*, 807–815. [PubMed]
139. Apás, A.; Arena, M.; Colombo, S.; González, S. Probiotic administration modifies the milk fatty acid profile, intestinal morphology, and intestinal fatty acid profile of goats. *J. Dairy Sci.* **2015**, *98*, 47–54. [CrossRef] [PubMed]
140. Kojima, M.; Suda, S.; Hotta, S.; Hamada, K.; Suganuma, A. Necessity of calcium ion for cell division in *Lactobacillus bifidus*. *J. Bacteriol.* **1970**, *104*, 1010–1013. [PubMed]

141. Duranti, S.; Milani, C.; Lugil, G.A.; Turroni, F.; Mancabelli, L.; Sanchez, B.; Ferrario, C.; Viappiani, A.; Mangifestra, M.; Mancino, W.; et al. Insights from genomes of representatives of the human gut commensal *Bifidobacterium bifidum*. *Environ. Microbiol.* **2015**, *17*, 2515–2531. [CrossRef] [PubMed]
142. Lugil, G.A.; Millani, C.; Turroni, F.; Duranti, S.; Ferrario, C.; Viappiani, A.; Mancabelli, L.; Mangifesta, M.; Taminiau, B.; Delcenserie, V.; et al. Investigation of the evolutionary development of the genus *Bifidobacterium* by comparative genomics. *Appl. Environ. Microbiol.* **2014**, *80*, 6383–6394. [CrossRef] [PubMed]
143. Milani, C.; Turroni, F.; Duranti, S.; Lugil, G.A.; Mancabelli, L.; Ferrario, C.; Sinderen, D.; Ventura, M. Genomics of the genus *Bifidobacterium* reveals species-specific adaptation to the glycan-rich gut environment. *Appl. Environ. Microbiol.* **2016**, *82*, 980–991. [CrossRef] [PubMed]
144. Ventura, M.; Canchaya, C.; Fitzgerald, G.F.; Gupta, R.S.; Sinderen, D. Genomics as a means to understand bacterial phylogeny and ecological adaptation: The case of bifidobacteria. *Antonie van Leeuwenhoek* **2007**, *91*, 351–372. [CrossRef] [PubMed]
145. Ventura, M.; O'Connell-Motherway, M.; Leahy, S.; Moreno-Munoz, J.A.; Fitzgerald, G.F.; Sinderen, D. From bacterial genome to functionality; case bifidobacteria. *Int. J. Food Mirobiol.* **2007**, *120*, 2–12. [CrossRef] [PubMed]
146. Turroni, F.; Bottacini, F.; Foroni, E.; Mulder, I.; Kim, J.H.; Zomer, A.; Sanchez, B.; Bidossi, A.; Ferrarini, A.; Giubellini, V.; et al. Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 19514–19519. [CrossRef] [PubMed]
147. Turroni, F.; Milani, C.; Duranti, S.; Mancabelli, L.; Mangifestra, M.; Viappiani, A.; Lugil, G.A.; Ferrario, C.; Gioiosa, L.; Ferrarini, A.; et al. Deciphering bifidobacterial-mediaated metabolic interactions and their impact of gut microbiota by a multi-omics approach. *ISME J.* **2016**, *10*, 1656–1668. [CrossRef] [PubMed]
148. Russell, D.A.; Ross, R.P.; Fitzgerald, G.F.; Stanton, C. Metabolic activities and probiotic potential of bifidobacteria. *Int. J. Food Microbiol.* **2011**, *149*, 88–105. [CrossRef] [PubMed]
149. Milani, C.; Lugil, G.A.; Duranti, S.; Turroni, F.; Bottacini, F.; Mangifestra, M. Genomic encyclopedia of type strains of the genus *Bifidobacterium*. *Appl. Environ. Microbiol.* **2014**, *80*, 6290–6302. [CrossRef] [PubMed]

Biochemical and Biophysical Research Communications 480 (2016) 222–227

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anti-obese effects of two *Lactobacilli* and two *Bifidobacteria* on ICR mice fed on a high fat diet

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ARTICLE INFO

Article history:
Received 7 October 2016
Accepted 13 October 2016
Available online 14 October 2016

Keywords:
Lactobacteria
Bifidobacteria
Anti-obesity
Fatty liver
Inflammation

ABSTRACT

Previous researchers have documented that probiotic bacteria can have anti-obesity effects on mice fed a high fat diet (HFD) and improve metabolic syndrome. The beneficial effects of the probiotic bacteria are suggested to be strain dependent. In this study, two candidate lactobacteria strains, *Lactobacillus casei* IBS041, *Lactobacillus acidophilus* AD031 and two bifidobacteria strains, *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI, were individually administered to HFD-fed mice for 8 weeks. *B. longum* BORI significantly suppressed mouse weight gain without affecting food intake. *L. acidophilus* and *B. bifidum* BGN4 significantly decreased triglyceride levels in mouse liver while *B. longum* BORI significantly lowered total cholesterol levels in liver. *L. acidophilus* and *B. bifidum* BGN4 significantly inhibited serum activities of aspartate transaminase and alanine transaminase. Diet supplementation with *L. acidophilus*, *B. bifidum* BGN4 and *B. longum* BORI efficiently improved hepatocyte hydropic degeneration and hepatic steatosis. Of the four probiotic candidates, the bifidobacteria *B. longum* BORI and *B. bifidum* BGN4, developed in our laboratory, and *L. acidophilus* AD031showed excellent anti-obesity effects and suppressed lipid deposition in liver.

1. Introduction

Obesity is one of the leading preventable causes of death worldwide, and increases the risk of developing various diseases, especially type 2 diabetes mellitus and cardiovascular diseases [1,2]. Gut microbiota was reported to be associated with metabolic homeostasis and obesity [3]. Intestinal bacteria can digest dietary carbohydrates to produce more short chain fatty acids (SCFA) such as acetate, propionate, butyrate and lactate and thereby increase energy harvest in the host organism [4]. Furthermore, such SCFA can play a role as metabolic regulators that can influence the expression of host genes in intestine and facilitate absorption of nutrients and differentiation of adipose tissue [5]. In the intestine of mice fed a high fat diet (HFD), more lipopolysaccharides (LPS) from gram-negative bacteria are incorporated into chylomicrons and absorbed by the host [6,7]. Moreover, LPS and some pathogens can disrupt tight junctions and increase intestinal permeability [8,9]. Elevated levels of LPS in serum, known as endotoxemia, results in obesity-coupled inflammation [10], which is related to metabolic syndrome, insulin resistance, and atherosclerosis [11,12]. In addition, long term HFD can lead to non-alcoholic hepatic steatosis, which is characterized by an excessive accumulation of triacylglycerol (TAG) within hepatocytes [13].

Despite controversy and criticism over the use of probiotics [14,15], they have been shown to have anti-obesity potential [16]. *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI, developed in our laboratory, have shown a variety of novel probiotic activities [17]. *B. bifidum* BGN4 has been documented to produce high amounts of S-adenosyl-L-methionine [18,19] and anticarcinogenic polysaccharides [20,21]. Moreover, it can suppress allergic responses [22–24], exert high adhesion to enterocyte-like Caco-2 cells [19], and prevent inflammatory bowel disease [25,26]. *B. longum* BORI is able to produce anti-rotavirus protein and reduce the severity of clinical symptoms in rotavirus-infected children [27]. In this study, we investigated the anti-obesity effects of these two bifidobacteria and compared them to those of two lactobacilli, *Lactobacillus casei* IBS041 and *Lactobacillus acidophilus* AD031 by using a HFD mouse model.

2. Materials and methods

2.1. Probiotic bacteria

Freeze-dried powder of *L. casei* (LC) IBS041, *L. acidophilus* (LA)

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http://dx.doi.org/10.1016/j.bbrc.2016.10.031
0006-291X/

AD031, *B. bifidum* BGN4 (BGN4) and *B. longum* BORI (BORI) were provided by Bifido Co., Ltd. (Hongchun, Korea).

2.2. Animals and diets

The ICR mice (age 7weeks), purchased from Central Lab. Animal (Seoul, Korea), were housed under a 12 h light/12 h dark cycle in a controlled room with a temperature of 23 ± 3 °C and a humidity of 50% ± 10%. After acclimating to the facility for 1 week, mice were randomly divided into 6 groups (n = 9) and fed a low fat diet (LFD; 10% of total calories from fat, Table 1), a HFD (60% of total calories from fat, Table 1), or a HFD supplemented with LC, LA, BGN4, or BORI probiotic powder for 8 weeks. Probiotic powder was suspended in drinking water at a density of 5×10^8 CFU/mL and the suspension was changed every second day. All mice were allowed free access to food and water. Mouse body weight and food intake were determined every second week. All animal-related procedures were approved by Institutional Animal Care and Use Committee of Seoul National University.

2.3. Histopathologic evaluation

After consuming an LFD or HFD for 8 weeks, mice underwent 12 h of fasting prior to being anaesthetized with Zoletil 50 (Virbac, Carros, France) and dissected. Blood samples were collected by heart punctures. Livers and epididymal fat pads were removed, and portions of each were fixed in 10% paraformaldehyde for subsequent H&E staining while the remaining portions stored at −80 °C for subsequent analyses.

2.4. Serological analyses

Serum levels of TAG, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and activity levels of aspartate transaminase (AST) and alanine transaminase (ALT) were determined by using test kits obtained from Asanpharm (Seoul, Korea). Low density lipoprotein cholesterol (LDL-C) levels were calculated by applying the formula LDL-C = TC − HDL-C − Triglycerides/5.

2.5. Hepatic lipid analyses

Total lipid was extracted from mouse liver by using the Folch method [28] with some modifications. Approximately 25 mg of liver was mixed with 20 fold of phosphate buffer saline and homogenized. Subsequently, 300 μL of the homogenate was mixed with 800 μL chloroform and 400 μL methanol and allowed to stand overnight at 4 °C. Afterward, 240 μL of 0.88% KCl solution was added and the mixture was vortexed vigorously and then centrifuged at 1000 *g* for 15 min. The lower centrifuged layer was evaporated under a hood and the residue was dissolved in 100 μL of isopropanol. Levels of TAG and TC were determined by using appropriate kits from Asanpharm.

2.6. Real-time polymerase chain reaction

Total RNA was extracted from adipose tissue by using an RNA extraction kit purchased from Takara Bio (Kusatsu, Japan). Afterward, 0.5 μg of total RNA from each sample were reverse-transcribed to cDNA by using a cDNA synthesis kit from Takara Bio. Relative quantifications of gene transcripts for TNF-α, IL-1β, and CD68 were completed by using SYBR premix from Takara Bio on a Applied Biosystems 7500 system. Primer sequences of GAPDH were: forward 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse 5′-TCC ACC ACC CTG TTG CTG TA-3'. Primer sequences of TNF-α were: forward 5′-TCT TCT CAT TCC TGC TTG TGG-3′, reverse 5′-GGT CTG GGG CAT AGA ACT GA-3'. Primer sequences of IL-1β were: forward 5′-GCC CAT CCT CTG TGA CTC AT-3′, reverse 5′-AGG CCA CAG GTA TTT TGT CG-3'. Primer sequences of CD68 were: forward 5′-TTC AGG GTG GAA GAA AGG TAA-3′, reverse 5′-CAA TGA TGA GAG GCA GCA AGA-3'. Relative mRNA levels were normalized to the GAPDH level and expressed as values of relative expression compared to that of the HFD group.

2.7. Statistical analysis

Results are expressed as mean ± standard error. Differences were examined by using one-way ANOVA followed by applying Duncan's multiple range tests. Statistical analyses used the SPSS statistical package (Chicago, IL, USA). Significance level of test results was set at $p < 0.05$.

3. Results

3.1. Effects of probiotic supplementation on food intake and weight parameters

Mice fed the LFD consumed more food than mice fed the HFD because of the low energy density of the LFD. Each of the four experimental groups (LC, LA, BGN4, and BORI) consumed approximately the same amount of food as that consumed by the HFD group, which indicates that probiotic supplementation did not significantly influence food intake (Fig. 1). However, all four experimental groups had a lower body weight than that of the HFD group (Fig. 2). In particular, supplementation of BORI significantly

Table 1
Formula of low fat diet and high fat diet.

LFD (10% calorie from fat)		HFD (60% calorie from fat)	
Formula	g/Kg	Formula	g/Kg
Casein	210.0	Casein	265.0
L-Cystine	3.0	L-Cystine	4.0
Corn Starch	280.0	Maltodextrin	160.0
Maltodextrin	50.0	Sucrose	90.0
Sucrose	325.0	Lard	310.0
Lard	20.0	Soybean Oil	30.0
Soybean Oil	20.0	Cellulose	65.5
Cellulose	37.15	Mineral Mix, AIN-93G-MX (94046)	48.0
Mineral Mix, AIN-93G-MX (94046)	35.0	Calcium Phosphate, dibasic	3.4
Calcium Phosphate, dibasic	2.0	Vitamin Mix, AIN-93-VX (94047)	21.0
Vitamin Mix, AIN-93-VX (94047)	15.0	Choline Bitartrate	3.0
Choline Bitartrate	2.75	Blue Food Color	0.1
Yellow Food Color	0.1		

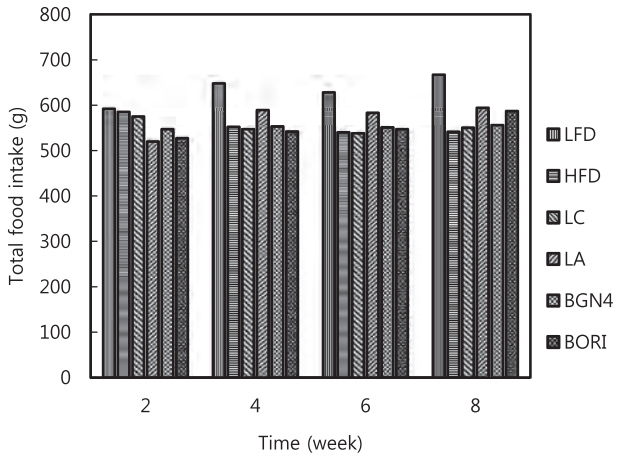

Fig. 1. Food intake of mice fed with a low fat diet or high fat diet.

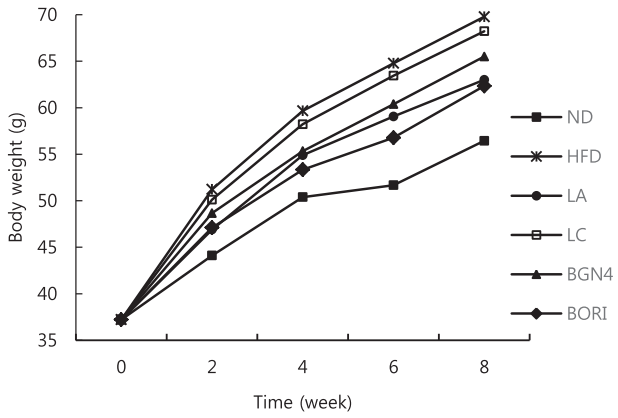

Fig. 2. Effect of probiotic bacteria on body weight of mice during the 8 weeks (n = 9).

suppressed mouse weight gain (Table 2). With regard to the food effect ratio (FER; *i.e.*, body weight gain per gram of food intake), all experimental groups had a lower FER value than that of the HFD group. The FER values of the LA and BORI groups were significantly lower than that of the HFD group. In addition, supplementation with BORI or LA significantly decreased liver weight. Supplementation with BGN4 ameliorated weight gain, FER, and liver weight (Table 2). However, epididymal fat pad weight was not influenced by supplementation with any of the four probiotic bacteria tested.

3.2. Effects of probiotic supplementation on serological parameters

The levels of serum lipid were not significantly different between HFD and LFD group. After 8 weeks of feeding, the levels of serum ALT and AST in the HFD group were significantly higher than those in the LFD group. ALT and AST serum levels were lower in LA and BGN groups than those in HFD group (Table 3).

3.3. Effects of probiotic supplementation on liver TAG and TC contents

After mice were fed the LFD or HFD for 8 weeks, the liver TAG and TC levels were determined. HFD intake increased the liver TAG and TC levels over those in mice fed the LFD. All probiotic supplemented groups showed lower TAG and TC levels than those in the HFD group. The groups supplemented with LA or BGN4 had significantly lower deposition of TAG in liver, whereas the BORI supplement group had a significantly lower TC level in liver (Fig. 3A, B, and C).

3.4. Effects of probiotic supplementation on inflammation of adipose tissue

As shown in Fig. 4A, all probiotic supplemented groups showed significantly smaller adipocyte sizes than that in the HFD group. Numerous “crown-like” structures are shown in the representative photograph of H&E-stained HFD group adipose tissue, indicating that numerous macrophages had infiltrated the adipose tissue. Regardless, macrophage infiltration was suppressed in all probiotic supplemented groups, but particularly in the LA, BORI, and BGN4 groups (Fig. 4B). Expression of CD68, a macrophage marker, was slightly suppressed in the four probiotic supplemented groups (Fig. 4C). Furthermore, expressions of cytokines TNF-α and IL-1β also showed a decrease in the four probiotic supplemented groups, particularly in the BGN4 group, though not statistically significantly (Fig. 4D and E).

Table 2
Effects probiotic bacteria on weight parameters of mice.

	LFD	HFD	LC	LA	BGN4	BORI
Original BW (g)	37.2 ± 3.5	37.2 ± 2.6	37.2 ± 2.4	37.2 ± 2.8	37.2 ± 2.2	37.2 ± 2.2
Final BW (g)	56.4 ± 7.8	69.8 ± 8.2	68.2 ± 10.9	63.0 ± 9.4	65.5 ± 8.5	62.3 ± 9.7
Weight gain (g)	19.2 ± 5.1^{b}	32.6 ± 6.7^{a}	31.0 ± 9.0^{a}	25.8 ± 9.0^{ab}	27.8 ± 7.6^{a}	25.1 ± 8.7^{b}
Food Intake (g)	2535	2218	2210	2286	2207	2203
FER (mg/g)	67 ± 9^{c}	132 ± 10^{a}	126 ± 29^{ab}	101 ± 21^{b}	112 ± 15^{ab}	102 ± 12^{b}
Liver (g)	2.2 ± 0.3^{b}	3.0 ± 0.6^{a}	2.6 ± 0.6^{ab}	2.4 ± 0.5^{b}	2.5 ± 0.5^{ab}	2.3 ± 0.5^{b}
Epididymal fat (g)	2.5 ± 0.8	3.2 ± 0.8	3.4 ± 0.9	3.0 ± ± 1.0	3.2 ± 0.5	2.9 ± 1.0

FER, food effect ratio = weight gain (mg)/food intake (g). ^{abc} Means not sharing a common letter are significantly different groups at $p < 0.05$.(n = 9).

Table 3
Effects probiotic bacteria on serological parameters of mice.

	LFD	HFD	LC	LA	BGN4	BORI
TAG (mg/dL)	110.9 ± 20.0	75.1 ± 11.7	83.8 ± 13.2	79.7 ± 17.3	72.2 ± 16.4	73.2 ± 20.4
TC (mg/dL)	134.7 ± 34.4	115.8 ± 30.2	107.7 ± 33.2	99.2 ± 28.3	107.1 ± 36.2	101.7 ± 35.3
LDL-c(mg/dL)	30.9 ± 17.5	17.5 ± 20.4	18.7 ± 36.4	32.8 ± 15.0	37.2 ± 28.7	16.8 ± 14.5
HDL-c(mg/dL)	88.5 ± 20.8	86.9 ± 14.0	64.9 ± 16.1	67.8 ± 20.7	74.7 ± 18.4	75.5 ± 25.5
ALT (IU/L)	5.1 ± 5.3^{b}	11.8 ± 6.5^{a}	5.5 ± 5.8^{b}	3.9 ± 3.4^{b}	5.8 ± 6.8^{b}	8.4 ± 3.9^{ab}
AST (IU/L)	13.1 ± 7.0^{b}	30.3 ± 12.1^{a}	19.9 ± 11.7^{ab}	17.1 ± 10.9^{b}	14.7 ± 6.9^{b}	22.3 ± 8.2^{ab}

^{abc} Means not sharing a common letter are significantly different groups at $p < 0.05$. (n = 7).

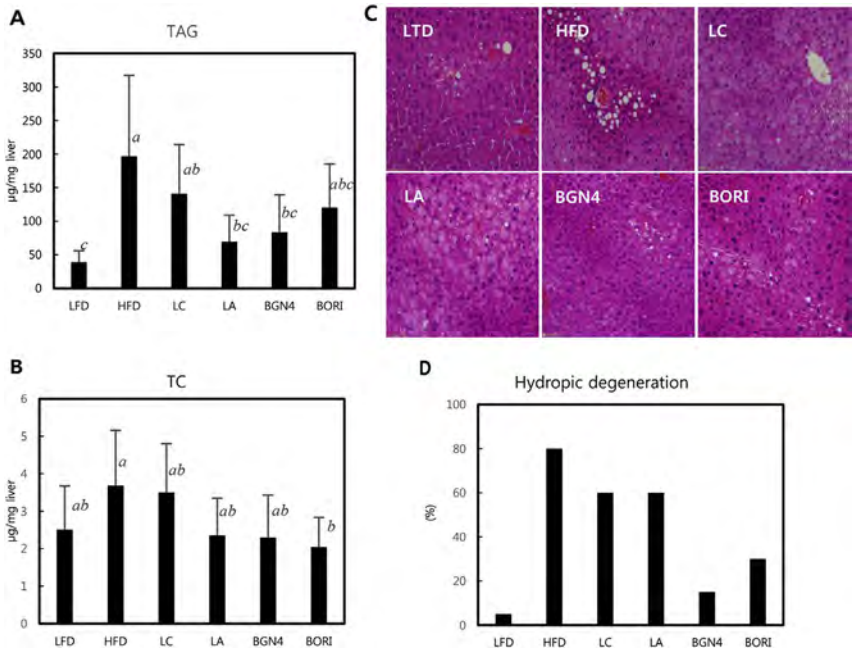

Fig. 3. Effects of probiotic bacteria supplementation on liver lipid contents and hepatocyte health. [abc] Means not sharing a common letter are significantly different groups at $p < 0.05$. (A) Content of TAG in the liver of mice ($n = 7$); (B) Content of TC in the liver of mice ($n = 7$); (C) H&E staining of mouse liver; (D) hydropic degeneration of hepatocytes. Hydropic degeneration area of liver was evaluated on the basis of the photograph of H&E staining in the way of double blind.

4. Discussion

Previous studies have reported that some strains of lactobacteria and bifidobacteria could suppress weight gain of murine fed a high fat diet [29–31]. In this work, *B. longum* BORI significantly inhibited weight gain in mice without affecting their level of food intake (Fig. 1 and Table 2). It has been reported that feces of obese mice contain less energy than that of lean mice [4]. Hamad et al. [32]

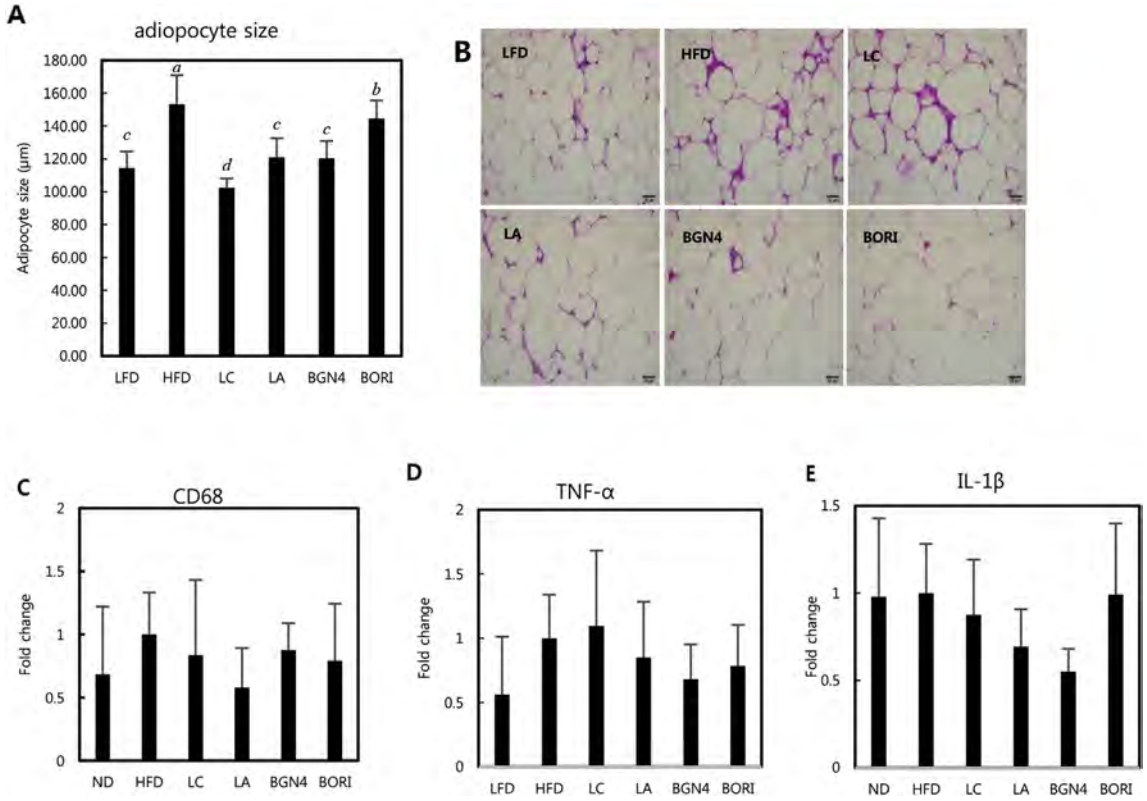

Fig. 4. Effects of probiotic bacteria supplementation on adipocyte size, macrophage recruitment and inflammation of adipose tissue. [abc] Means not sharing a common letter are significantly different groups at $p < 0.05$. (A) adipocyte size; (B) H&E staining of adipose tissue; (C) relative expression of CD68 in adipose tissue ($n = 5$); (D) relative expression of TNF-α in adipose tissue ($n = 5$); (E) relative expression of IL-1β in adipose tissue ($n = 5$).

reported a decrease in the maximum lymphatic absorption of TAG, phospholipids, and cholesterol in rats fed milk fermented by *Lactobacillus gasseri* SBT2055. Since different intestinal bacteria may degrade different food substances, simplification in the kinds of bacteria within the gut microbiota might decrease the total energy harvest of that host.

In support of previous studies [33,34], we observed that *B. longum* BORI and *L. acidophilus* significantly suppress an increase in liver weight (Table 2), a result that is attributed to less fat being deposited in liver. As shown in the photographs in Fig. 3C, livers of mice fed with probiotic supplements had fewer white fat droplets than that in the HFD group. In addition, Fig. 3A shows that the TAG levels in the probiotic supplemented groups are lower than that in the HFD group.

Non-alcoholic fatty liver disease (NAFLD) occurs when fat is deposited in the liver due to causes other than excessive alcohol use, and NAFLD is the most common liver disorder in developed countries [35,36]. Metabolic disorders such as lipodystrophy result in hydropic degeneration of hepatocytes, which, in this study, was significantly lowered by feeding with a probiotic supplement (Fig. 3D). Both ALT and AST levels are commonly measured clinically as a part of a diagnostic evaluation of hepatocellular injury in order to determine liver health [37,38]. In the present study, ALT and AST activity levels in the probiotic supplemented groups were lower than that of the HFD group (Table 3). Furthermore, as shown in Fig. 3B, the liver TC levels of mice supplemented with probiotics were lower than that of mice in the HFD group. Probiotic bacteria exerting bile acid hydrolase activity can increase fecal bile acid deconjugation and excretion as well as hepatic bile acid neosynthesis in mice [39–41], and cholesterol is used to synthesize new bile acids in a homeostatic response, resulting in a lowering of hepatic cholesterol levels [40].

In previous studies, probiotic bacteria were shown to suppress increases of plasma TAG and cholesterol induced by consuming a HFD [34,42]. However, these effects were not observed in our study (Table 3).

Certain lactobacteria and bifidobacteria have been reported to reduce adipocyte size in HFD-fed mouse model [30,43]. This effect was also observed in the present study (Fig. 4A). All four probiotic bacteria assessed in this study (*L. casei*, *L. acidophilus*, *B. bifidus* BGN4 and *B. longum* BORI) significantly reduced adipocyte size of mice fed the HFD.

When fed a HFD, more LPS are incorporated into chylomicrons and absorbed into the circulatory system, which leads to chronic low-grade inflammation. Increased necrosis-like adipocyte cell death in the obesity is probably due to the detrimental effects of adipocyte hypertrophy and may result in recruitment of macrophages to adipose tissue [44]. Dead adipocytes are often found surrounded by macrophages in so-called “crown-like” structures and are thought to scavenge cell debris and free lipid droplets [45]. In the photographs showing our H&E staining results (Fig. 4B), there are more “crown-like” structures in the HFD group and fewer such structures in the LA, BGN4, and BORI groups.

Expression levels of CD68 in the probiotic supplemented groups were lower than that in the HFD group, although the difference was not significant (Fig. 4C). In addition, the probiotic supplemented groups showed a pattern of inhibiting the mRNA levels of the TNF-α and IL-1β cytokines, which was especially apparent in the BGN4 group (Fig. 4D and E).

Certain kinds of probiotics tightly adhere to the intestinal mucosa during their colonization of the intestinal tract, a gut region where they repetitively compete with other bacteria, such as enteropathogens [46,47]. Probiotic bacteria are reported to enhance expressions of occludin and ZO-1, and they maintain the integrity of tight junctions [48,49]. Through these actions, endotoxemia may be reduced, which can be of benefit in instances of systemic chronic inflammation and metabolism syndrome.

In summary, *B. longum* BORI, *L. acidophilus*, and *B. bifidum* BGN4 can efficiently suppress weight gain and liver fat deposition in mice, and those supplements have a tendency to inhibit macrophage recruitment and cytokine release.

Conflict of interest

Geun Eog Ji is a professor of Seoul National University and also a president of Bifido Co., Ltd. The rest of authors declare no conflict of interest.

Acknowledgment

This work was supported by Cooperative Research Program for Agriculture Science & Technology Development, Rural Development Administration, Republic of Korea (Project No. PJ01123001); and the Promoting Regional specialized Industry, the Ministry of Trade, Industry and Energy (MOTIE) and Korea Institute for Advancement of Technology (KIAT), Republic of Korea (Project No. R0004140).

References

[1] L.A. Barness, J.M. Opitz, E. Gilbert-Barness, Obesity: genetic, molecular, and environmental aspects, Am. J. Med. Genet. 143 (2007) 3016–3034.
[2] S.M. Grundy, Obesity, metabolic syndrome, and cardiovascular disease, J. Clin. Endocr. Metlab. 89 (2004) 2595–2600.
[3] N.M. Delzenne, A.M. Neyrinck, F. Bäckhed, P.D. Cani, Targeting gut microbiota in obesity: effects of prebiotics and probiotics, Nat. Rev. Endocrinol. 7 (2011) 639–646.
[4] P.J. Turnbaugh, R.E. Ley, M.A. Mahowald, V. Magrini, E.R. Mardis, J.I. Gordon, An obesity-associated gut microbiome with increased capacity for energy harvest, Nature 444 (2006) 1027–1131.
[5] B.S. Samuel, A. Shaito, T. Motoike, F.E. Rey, F. Backhed, J.K. Manchester, R.E. Hammer, S.C. Williams, J. Crowley, M. Yanagisawa, Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41, P. Natl. Acad. Sci. 105 (2008) 16767–16772.
[6] S. Ghoshal, J. Witta, J. Zhong, W. De Villiers, E. Eckhardt, Chylomicrons promote intestinal absorption of lipopolysaccharides, J. Lipid Res. 50 (2009) 90–97.
[7] F. Laugerette, C. Vors, A. Géloën, M.-A. Chauvin, C. Soulage, S. Lambert-Porcheron, N. Peretti, M. Alligier, R. Burcelin, M. Laville, Emulsified lipids increase endotoxemia: possible role in early postprandial low-grade inflammation, J. Nutr. Biochem. 22 (2011) 53–59.
[8] H. Yan, Butyrate increases tight junction protein expression and enhances tight junction integrity in porcine IPEC-J2 cells stimulated with LPS, in: 2016 Joint Annual Meeting, Asas, 2016.
[9] J.A. Guttman, Y. Li, M.E. Wickham, W. Deng, A.W. Vogl, B.B. Finlay, Attaching and effacing pathogen-induced tight junction disruption in vivo, Cell. Microbiol. 8 (2006) 634–645.
[10] S. Chirumbolo, G. Franceschetti, E. Zoico, C. Bambace, L. Cominacini, M. Zamboni, LPS response pattern of inflammatory adipokines in an in vitro 3T3-L1 murine adipocyte model, Inflamm. Res. 63 (2014) 495–507.
[11] E. Maury, S. Brichard, Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome, Mol. Cell. Endocrinol. 314 (2010) 1–16.
[12] U. Kintscher, M. Hartge, K. Hess, A. Foryst-Ludwig, M. Clemenz, M. Wabitsch, P. Fischer-Posovszky, T.F. Barth, D. Dragun, T. Skurk, T-lymphocyte infiltration in visceral adipose tissue a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance, Atertio. Thromb. Vasc. Biol. 28 (2008) 1304–1310.
[13] M.-S. Gauthier, R. Favier, J.-M. Lavoie, Time course of the development of non-alcoholic hepatic steatosis in response to high-fat diet-induced obesity in rats, Br. J. Nutr. 95 (2006) 273–281.
[14] K.L. Lindsay, M. Kennelly, M. Culliton, T. Smith, O.C. Maguire, F. Shanahan, L. Brennan, F.M. McAuliffe, Probiotics in obese pregnancy do not reduce maternal fasting glucose: a double-blind, placebo-controlled, randomized trial (Probiotics in Pregnancy Study), Am. J. Clin. Nutr. 99 (2014) 1432–1439.
[15] J. Štšepetova, E. Sepp, H. Kolk, K. Loivukene, E. Songisepp, M. Mikelsaar, Diversity and metabolic impact of intestinal Lactobacillus species in healthy adults and the elderly, Br. J. Nutr. 105 (2011) 1235–1244.
[16] T. Arora, S. Singh, R.K. Sharma, Probiotics: interaction with gut microbiome and antiobesity potential, Nutrition 29 (2013) 591–596.
[17] S. Ku, M.S. Park, G.E. Ji, H.J. You, Review on Bifidobacterium bifidum BGN4: functionality and nutraceutical applications as a probiotic microorganism, Int.

J. Mol. Sci. 17 (2016) 1544.
[18] J.-Y. Kim, J.-W. Suh, G.-E. Ji, Evaluation of S-adenosyl-L-methionine production by Bifidobacterium bifidum BGN4, Food Sci. Biotechnol. 17 (2008) 184–187.
[19] J.Y. Kim, H.-S. Seo, M.J. Seo, J.-W. Suh, I. Hwang, G.E. Ji, Development of S-adenosyl-L-methionine (SAM)-reinforced probiotic yogurt using Bifidobacterium bifidum BGN4, Food Sci. Biotechnol. 17 (2008) 1025–1031.
[20] H.J. You, D.-K. Oh, G.E. Ji, Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from Bifidobacterium bifidum BGN4, FEMS Microbiol. Lett. 240 (2004) 131–136.
[21] S. Ku, H.J. You, G.E. Ji, Enhancement of anti-tumorigenic polysaccharide production, adhesion, and branch formation of Bifidobacterium bifidum BGN4 by phytic acid, Food Sci. Biotechnol. 18 (2009) 749–754.
[22] H. Kim, S.-Y. Lee, G.E. Ji, Timing of Bifidobacterium administration influences the development of allergy to ovalbumin in mice, Biotechnol. Lett. 27 (2005) 1361–1367.
[23] H. Kim, K. Kwack, D.-Y. Kim, G.E. Ji, Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model, FEMS Immunol. Med. Microbiol. 45 (2005) 259–267.
[24] S.Y. Lee, S.K. Oh, S.W. Park, G.R. Jeon, J.Y. Kim, S.Y. Yoon, G.E. Ji, Evaluation of anti-allergic effect of bifidobacteria in murine model of peanut allergy, Pediatr. Allergy Respir. Dis. 16 (2006) 131–141.
[25] N. Kim, J. Kunisawa, M.-N. Kweon, G.E. Ji, H. Kiyono, Oral feeding of Bifidobacterium bifidum (BGN4) prevents CD4+ CD45RB high T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation, Clin. Immunol. 123 (2007) 30–39.
[26] J.Y. Kim, K.S. Hong, J.S. Kim, K.M. Ahn, G.E. Ji, Clinical effect of Bifidobacterium bifidum BGN4-containing probiotic products on the suppression of atopy and irritable bowel syndrome, in: 2009 년도 International Meeting of the Microbiological Society of Korea, May, 2009, pp. 44–45.
[27] G.E. Ji, Development of Bifidobacterium sp. BGN4 and BORI with novel probiotic activity, in: 2005 년도 International Meeting of the Microbiological Society of Korea, May, 2005, pp. 81–84.
[28] J. Folch, M. Lees, G. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[29] H.M. An, S.Y. Park, D.K. Lee, J.R. Kim, M.K. Cha, S.W. Lee, H.T. Lim, K.J. Kim, N.J. Ha, Antiobesity and lipid-lowering effects of Bifidobacterium spp. in high fat diet-induced obese rats, Lipids Health Dis. 10 (2011) 1.
[30] J. Wang, H. Tang, C. Zhang, Y. Zhao, M. Derrien, E. Rocher, J.E.v.-H. Vlieg, K. Strissel, L. Zhao, M. Obin, Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice, ISME J. 9 (2015) 1–15.
[31] S.-W. Kim, K.-Y. Park, B. Kim, E. Kim, C.-K. Hyun, Lactobacillus rhamnosus GG improves insulin sensitivity and reduces adiposity in high-fat diet-fed mice through enhancement of adiponectin production, Biochem. Biophys. Res. Commun. 431 (2013) 258–263.
[32] E.M. Hamad, M. Sato, K. Uzu, T. Yoshida, S. Higashi, H. Kawakami, Y. Kadooka, H. Matsuyama, I.A.A. El-Gawad, K. Imaizumi, Milk fermented by Lactobacillus gasseri SBT2055 influences adipocyte size via inhibition of dietary fat absorption in Zucker rats, Br. J. Nutr. 101 (2009) 716–724.
[33] X. Ma, J. Hua, Z. Li, Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells, J. Hepatol. 49 (2008) 821–830.
[34] E. Esposito, A. Iacono, G. Bianco, G. Autore, S. Cuzzocrea, P. Vajro, R.B. Canani, A. Calignano, G.M. Raso, R. Meli, Probiotics reduce the inflammatory response induced by a high-fat diet in the liver of young rats, J. Nutr. 139 (2009) 905–911.
[35] M. Shaker, A. Tabbaa, M. Albeldawi, N. Alkhouri, Liver transplantation for nonalcoholic fatty liver disease: new challenges and new opportunities, World J. Gastroenterol. WJG 20 (2014) 5320.
[36] M.E. Rinella, Nonalcoholic fatty liver disease: a systematic review, JAMA 313 (2015) 2263–2273.
[37] C.-S. Wang, T.-T. Chang, W.-J. Yao, S.-T. Wang, P. Chou, Impact of increasing alanine aminotransferase levels within normal range on incident diabetes, J. Formos. Med. Assoc. 111 (2012) 201–208.
[38] N. Ghouri, D. Preiss, N. Sattar, Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data, Hepatology 52 (2010) 1156–1161.
[39] M.L. Jones, C. Tomaro-Duchesneau, S. Prakash, The gut microbiome, probiotics, bile acids axis, and human health, Trends Microbiol. 22 (2014) 306–308.
[40] M. Begley, C. Hill, C.G. Gahan, Bile salt hydrolase activity in probiotics, Appl. Environ. Microbiol. 72 (2006) 1729–1738.
[41] C. Degirolamo, S. Rainaldi, F. Bovenga, S. Murzilli, A. Moschetta, Microbiota modification with probiotics induces hepatic bile acid synthesis via down-regulation of the Fxr-Fgf15 axis in mice, Cell Rep. 7 (2014) 12–18.
[42] S. Kondo, J.-Z. Xiao, T. Satoh, T. Odamaki, S. Takahashi, H. Sugahara, T. Yaeshima, K. Iwatsuki, A. Kamei, K. Abe, Antiobesity effects of Bifidobacterium breve strain B-3 supplementation in a mouse model with high-fat diet-induced obesity, Biosci. Biotechnol. Biochem. 74 (2010) 1656–1661.
[43] N. Takemura, T. Okubo, K. Sonoyama, Lactobacillus plantarum strain No. 14 reduces adipocyte size in mice fed high-fat diet, Exp. Biol. Med. 235 (2010) 849–856.
[44] S. Cinti, G. Mitchell, G. Barbatelli, I. Murano, E. Ceresi, E. Faloia, S. Wang, M. Fortier, A.S. Greenberg, M.S. Obin, Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans, J. Lipid Res. 46 (2005) 2347–2355.
[45] M. Zeyda, T.M. Stulnig, Adipose tissue macrophages, Immunol. Lett. 112 (2007) 61–67.
[46] I.H. Kim, M.S. PARK, G.E. Ji, Characterization of adhesion of Bifidobacterium sp. BGN4 to human enterocyte-like Caco-2 cells, J. Microbiol. Biotechnol. 13 (2003) 276–281.
[47] M. Candela, F. Perna, P. Carnevali, B. Vitali, R. Ciati, P. Gionchetti, F. Rizzello, M. Campieri, P. Brigidi, Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production, Int. J. Food Microbiol. 125 (2008) 286–292.
[48] R. Ranuh, M. Subijanto, I. Surono, The Role of probiotic lactobacillus plantarum IS 20506 on Occludin and ZO-1 of intestinal tight junctions rehabilitation, Adv. Nat. Appl. Sci. 7 (2013) 480–484.
[49] H. Putaala, T. Salusjärvi, M. Nordström, M. Saarinen, A.C. Ouwehand, E.B. Hansen, N. Rautonen, Effect of four probiotic strains and Escherichia coli O157: H7 on tight junction integrity and cyclo-oxygenase expression, Res. Microbiol. 159 (2008) 692–698.

The Journal of Microbiology (2012) Vol. 50, No. 4, pp. 638–643
DOI 10.1007/s12275-012-1591-x

Novel *Bifidobacterium* Promoters Selected Through Microarray Analysis Lead to Constitutive High-Level Gene Expression

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(Received December 7, 2011 / Accepted April 17, 2012)

For the development of a food-grade expression system for *Bifidobacterium*, a strong promoter leading to high-level expression of cloned gene is a prerequisite. For this purpose, a promoter screening host-vector system for *Bifidobacterium* has been established using β-glucosidase from *Bifidobacterium lactis* as a reporter and *Bifidobacterium bifidum* BGN4 as a host, which is β-glucosidase negative strain. Seven putative promoters showing constitutive high-level expression were selected through microarray analysis based on the genome sequence of *B. bifidum* BGN4. They were cloned into upstream of β-glucosidase gene and transformed into *Escherichia coli* DH5α and *B. bifidum* BGN4. Promoter activities were analyzed both in *E. coli* and *B. bifidum* BGN4 by measuring β-glucosidase activity. β-Glucosidase activities in all of the transformants showed growth-associated characteristics. Among them, P919 was the strongest in *B. bifidum* BGN4 and showed maximum activity at 18 h, while P895 was the strongest in *E. coli* DH5α at 7 h. This study shows that novel strong promoters such as P919 can be used for high-level expression of foreign genes in *Bifidobacterium* and will be useful for the construction of an efficient food-grade expression system.

Keywords: *Bifidobacterium*, promoter, expression, vector

Introduction

Bifidobacterium is a strictly anaerobic Gram-positive bacterium with high guanine and cytosine content and often Y-shaped or clubbed morphology. They all contain fructose-6-phosphate phosphoketolase, which can be used as a differential marker for the genus *Bifidobacterium* (Bezkorovainy and Miller-Catchpole, 1989; Mitsuoka, 1990). *Bifidobacterium* is a major commensal bacterium in the intestines of humans and animals. It is considered to play a beneficial role in the maintenance of the balance of normal intestinal flora in humans (Park *et al.*, 2008).

Ever since the first report on the existence of plasmids in the genus *Bifidobacterium* (Sgorbati *et al.*, 1982), continuous progress has been made in the development of cloning vectors for *Bifidobacterium* to improve its probiotic characteristics (Kullen and Klaenhammer, 2000). A high-level expression system requires a strong promoter to express foreign genes in a host. Many *Escherichia coli* promoters have been reported to contain a -10 region similar to those of *Lactobacillus acidophilus* promoters (Kullen and Klaenhammer, 2000), but they cannot be used in foods because of their non-food-grade nature. Therefore, the development of food-grade expression systems has been pursued in various studies using genetic elements derived from food-grade microorganisms (Kim *et al.*, 2009). For this purpose, the screening of strong promoters from food-grade microorganisms is an important research area (Kim *et al.*, 2009). However, only a few studies have reported the characterization of promoters from the genus *Bifidobacterium*. Park *et al.* (2008) constructed an expression vector using a 16S rRNA promoter of *Bifidobacterium longum* and expressed cholesterol oxidase from *Streptomyces* in *B. longum*. Klijn *et al.* (2006) used the *gusA* gene of *E. coli* as a reporter and characterized 3 promoters from *B. longum* using microarray analysis.

Reporter genes are generally used to select recombinant plasmids and characterize promoter strength in various bacterial strains (Sirard *et al.*, 1995; Gibson and Tabita, 1996; Rist and Kertesz, 1998).

In this report, we constructed a promoter screening host-vector system for *Bifidobacterium*. The structural gene of β-glucosidase from *Bifidobacterium lactis* AD011 was cloned into pBES2, a *Bifidobacterium–E. coli* shuttle vector, and β-glucosidase–free *Bifidobacterium bifidum* BGN4 was used as a host. Seven putative promoters selected on the basis of microarray analysis of *B. bifidum* BGN4 were then cloned and tested for their promoter activities.

Materials and Methods

Bacterial strains, media, and plasmids

E. coli DH5α was grown in Luria-Bertani broth (Difco, USA) at 37°C with vigorous shaking. *B. bifidum* BGN4 was grown in MRS medium (Difco) supplemented with 0.05% (w/v) L-cysteine·HCl at 37°C. Ampicillin and chloramphenicol (Sigma, USA) were used at concentrations of 50 and 3.6 μg/ml, respectively, for transformant selection. The bacterial strains and plasmids used in this work are listed in Table 1.

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	Cloning host	Hanahan (1983)
B. bifidum BGN4	β-glucosidase negative source of promoters	
Plasmids		
pBES2	Cm^r, Amp^r: *E. coli-Bifidobacterium* shuttle vector	Park *et al.* (2003)
pBES2-16pG	pBES2 derivative containing 16S rRNA promoter from *Bifidobacterium longum* upstream of β-glucosidase	Park *et al.* (2008)
pBES2-G	Promoter screening vector for *Bifidobacterium* containing promoterless β-glucosidase gene	This work (Fig. 1)
pGEM-T easy	Amp^r, M13ori pBR322ori, linear T-overhangs vector	Promega
pBES2-632G	pBES2 derivative containing the P632 promoter upstream of β-glucosidase	This work
pBES2-644G	pBES2 derivative containing the P644 promoter upstream of β-glucosidase	This work
pBES2-895G	pBES2 derivative containing the P895 promoter upstream of β-glucosidase	This work
pBES2-834G	pBES2 derivative containing the P834 promoter upstream of β-glucosidase	This work
pBES2-888G	pBES2 derivative containing the P888 promoter upstream of β-glucosidase	This work
pBES2-919G	pBES2 derivative containing the P919 promoter upstream of β-glucosidase	This work
pBES2-1527G	pBES2 derivative containing the P1527 promoter upstream of β-glucosidase	This work

General cloning techniques and vector construction

The chromosomal DNA of *B. bifidum* BGN4 was isolated using a PureLink™ Genomic DNA Kit (Invitrogen, USA) according to the manufacturer's instructions and used as a template for polymerase chain reaction (PCR) amplification. Seven genomic DNA regions predicted to have promoter activity were amplified using each primer set containing *Xba*I and *Bam*HI restriction sites (Table 2). PCR products were ligated into a pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α using the $CaCl_2$ method (Sambrook *et al.*, 1989). Plasmid DNA was isolated from *E. coli* DH5α using a Plasmid Purification Mini Kit (Nucleogen, Korea) according to the manufacturer's instructions. DNA fragments were purified from the agarose gel using a gel extraction kit (QIAGEN Korea Ltd., Korea). All restriction enzymes were purchased from Promega.

Microarray analysis

A total of 1,000 probes were designed based on the genome sequence data of *B. bifidum* BGN4 (not published), and Combimatrix customized chips for these genes were designed and manufactured by Macrogen Inc. (Korea). *B. bifidum* BGN4 was grown in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl with or without controlling the pH at 5.0 with 4 N NaOH. A 5-L jar fermenter (Hanil Inc., Korea) was used to cultivate the *B. bifidum* BGN4. During cultivation, 1.5 ml of culture broth was harvested at the indicated time points and the total RNA was extracted using a RiboPure™ Bacteria Kit (Ambion Inc., USA) according to the manufacturer's instructions. Microarray analysis was performed at Macrogen Inc.

Transformation of *Bifidobacterium*

Expression vectors were prepared from *E. coli* DH5α using an Axyprep Midi Kit (Axygen Biosciences, USA) and methylated using GpC methyltransferase (M.CviPI; NEB, USA) according to the manufacturer's instructions. After methylation, the expression vectors were transformed into *B. bifidum* BGN4 by electroporation according to Kim *et al.* (2010). The fructose-6-phosphate phosphoketolase test and plasmid preparation was conducted to confirm the correct transformants among the developed colonies.

Sequence analysis of the putative promoter

DNA sequence analysis was performed using the Applied Biosystems 3730 DNA Analyzer at the Genome Research Facility of Seoul National University.

The promoter regions and transcription starting points of 7 putative promoters were predicted using the program at http://www.fruitfly.org/seq_tools/promoter.html. The minimum promoter score was fixed at 0.7.

Promoter activity assay

The β-glucosidase activities of recombinant *E. coli* DH5α and *B. bifidum* BGN4 were analyzed using *p*-nitrophenyl-β-D-glucopyranoside as a substrate to compare the promoter

Table 2. Primers for amplifying the putative promoters from *B. bifidum* BGN4

Primer names	Sequences of primers	
	Forward	Reverse
P632	5′-tctagaACGAATAGGCAGGCGTTGCTG -3′	5′-ggatccGCTGGCTCCTTTGTTTGCGTA-3′
P644	5′-tctagaCACGCGCAGCTGTTTTGAAG-3′	5′-ggatccGTAATTGTCCTCCTGGACGT-3′
P895	5′-tctagaATCGTACAGCACGAAAACCGT-3′	5′-ggatccTGCCGTCCGCCCTTTCTAGCG-3′
P834	5′-tctagaTTTTCGGCGGGTCTTCGGTCC-3′	5′-ggatccCATCGAGGCATGATTGTAGCA-3′
P888	5′-tctagaTTGCCGCTGCCGGTCGTATCG-3′	5′-ggatccAGCCTTACAGTCCATTCCTTG-3′
P919	5′-tctagaTGAAGTGTGTCGTGTGGCGT-3′	5′-ggatccTGGTGTACCTTTTCTTGCTT-3′
P1527	5′-tctagaGCCTCGATGGCGGCTTCGGG-3′	5′-ggatccAATGGCTCTCCTTGTAATAC-3′

Forward and reverse primers were designed to contain *Xba*I (tctaga) and *Bam*HI (ggatcc) sites, respectively

Table 3. List of genes with high-level median signal values in microarray data for *B. bifidum* BGN4

Gene names	Gene Comments[a]	Median signal values[b]							
		With pH control				Without pH control			
		8[c]	9	10	11	8	9	10	11
644	Tuf	65535	65535	65535	65535	65535	65535	65535	65535
1527	rpsP	65535	65535	65535	65535	65535	65535	65535	65535
919	rplM	9911.5	16664.5	15641	15974.5	17120	16380	25741	16061.5
888	rpmJ	53734	54808.5	50175.5	45308.5	61536	65535	63081	59946
632	ybhL	58367.5	56733.5	53298	59489.5	55920	60428.5	65535	57233.5
834	cell wall	51138	41770.5	54758	48567.5	52433	57057	65535	58731
895	rplR	18737	20172.5	10552.5	15758.5	17589.5	16451.5	19626	9422.5

[a] Tuf, peptide elongation factor Tu; rps, ribosomal protein small; rpl, ribosomal protein large; rpm, ribosomal protein medium; ybh, putative inner membrane protein; cell wall, cell wall biogenesis
[b] The maximal median signal value was set at 65535 and the bigger signals were also indicated as 65535
[c] 8, 10 min before NaOH input; 9, 40 min after NaOH input; 10, 70 min after NaOH input; 11, 160 min after NaOH input

strengths.

B. bifidum BGN4 and *B. lactis* AD011 were cultured in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl at 37°C. Nine *B. bifidum* BGN4 transformants harboring pBES2-632G, pBES2-644G, pBES2-895G, pBES2-834G, pBES2-888G, pBES2-919G, pBES2-1527G, pBES2-G, and pBES2-16pG were cultivated in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl and 3.6 μg/ml of chloramphenicol at 37°C.

E. coli DH5α transformants (pBES2-632G, pBES2-644G, pBES2-895G, pBES2-834G, pBES2-888G, pBES2-919G, pBES2-1527G, pBES2-G, and pBES2-16pG) and *E. coli* DH5α were cultured in LB medium at 37°C with vigorous shaking, and ampicillin was used at 50 μg/ml as necessary.

During cultivation, 1.0 ml of culture broth was harvested at indicated times and centrifuged at 10,000×*g* for 2 min. The cell pellet was washed twice with 200 μl of ice-cold 20 mM phosphate buffer (pH 5.0), disrupted by sonication in 500 μl of the same buffer, and centrifuged at 10,000×*g* for 2 min at 4°C to obtain the supernatant as crude enzyme. Fifteen microliters of enzyme solution was mixed with 10 μl of 5 mM *p*-nitrophenyl-β-D-glucopyranoside and incubated at 37°C for 20 min. The reaction was stopped by the addition of 100 μl of 1 M Na_2CO_3. The released *p*-nitrophenol (*p*NP) was measured at 405 nm using *p*NP (Sigma) as a standard. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol (*p*NP) per min at 37°C.

Results

Microarray analysis of *B. bifidum* BGN4

When *B. bifidum* BGN4 was grown in MRS medium, the pH decreased and reached 5.0 after 8 h, 10 min. Subsequently, the pH of the one reactor was controlled at 5.0 by 4 N NaOH, while the other was not. The pH controlled reactor showed better growth and reached higher optical density after 10 h, 50 min. In this study, we attempted to obtain promoters of *Bifidobacterium* showing constitutive high-level expression. Microarray data analysis revealed 7 genes showing constitutively high-level expression in both culture conditions (Table 3). Among them, 644 and 1527 were the strongest, 919 and 895 were the weakest, and 888, 632, and

Table 4. Nucleotide sequences of the putative promoters from *B. bifidum* BGN4

Promoter (size)	Promoter sequences
P632 (102 bp)	Acgaataggcaggcgttgctggtgtgatgctcaaggctgaacattaatgatttgttcagccttctccgttacga***TG***aagc**G**tacgcaaaca**aaggag**ccagc (0.74)
P834 (180 bp)	Ttttcggcgggtcttcggtccccgccagtgagattcatctctattattctcccgggagtgtcgcgccggtcgtgtgaagattgccggttttcccgtgatgt***TG***ccga**T**atggcgtgatttcctagcgtgagt gttttcaggcg**aaga**tgcatgcctgtgtgctacaatcatgcctcgatg (0.73)
P895 (526 bp)	Atcgtacagcacgaaaaccgtgcgcggggccgtttggctactgcctttcccgatagcccattgaaaatcgtgtacggataggtgtatcggcgcactgagaggcgagaataatgggctatctgcatttcga ggggttgatggcagagtagaggcgctccgggaaccgcatgattccaacaaccgtagggcagtgataccgaggggatcgtgctctgacaacctcacgaaacgcagatagcgccgaggggcggcagcc ctgaggggatatgacaggattcgggcgtgcgacacgcccgaattccctataggtacccctggtgctttaattaacaagttgcctgttttgggctcgcaaattggaa***T***caaaa**A**agcgagcgtggaacaa ggggtccgtttcgacgggcttcttgcatgtctacgcactgatgtggttatggccgcgaaaagtattttcgtgtgtcatgctcgccggtgccctttagc**agga**tggtaatagtcaacgaatcgct**agaaaggg** cggacggca (0.72)
P644 (173 bp)	Cacgcgcagctgttttgaagccgtaagtgtctccagtcagcggtaaaatcatataccgctgactgggttctggctccaaagtggcaagaaacccagaaacccagtacaa***TG***tagc**G**agtgtcttgtgc cgcaagcgcaagccaactacacgagacgtccaggaggacaattac (0.97)
919 (206 bp)	Tgaagtgtgtcgtggcgttgcgaaatgtcaaaggtggcgttatca***T***agagg**A**ttgttgtgtttccctaaggggtccttcaaagcgctttcccactcgccatcgaggactttcagggagcccacggata atgaggccgaaaccgccaagcctgaccagcgcggcagtccaagcccggaacacaataagcaagaaaaggtacacca (0.81)
P1527 (576 bp)	Gcctcgatggcggcttcgggctggacagcgtccaagcaaacccgccgttatccgtatcgaaaccgcccgctgcgaccggccaaggtaccccatgtggccgtatctcgttttcaaggggttgatttcggct ccgccggacatctatctgcgtttcaaggggcgccaaaacgcgatatccgaagtatgatcggcgaaataccaacggaatcatgcggttcttgggccacttatactttgggaccaacccctcgaaacgcag ataggcattcggaacaacacgaatcaacccctcgaaatgcagatagccccgaatcctccgccagtgaatgccgaccaggctttcaagcatcacgtcagacaccttgcgcgccgatgtttttgccacatcg accccgacgatcgctcgccagctccagcgtgtcgggtatccaccccctatgcgttataatttatcggttattcgtgatgggtggagccctctcattctgcctttcatgaggaacacgtgggttctcggttgagg tgccccacacacaatcgaaaa***T***ccgca**A**acctagtattac**aaggag**agccatt (0.90)
P888 (164 bp)	Ttgccgctgccggtcgtatcgccggcaggagcaaggataaagcaagaaattataacgggtcatgcacgcctcggcgtgtcgtgacccgttatgatatattctttaaatttggtg**T**gtcttcggacatacaa g***TG***taa**T**acaccca**Agga**atggactgt**aagg**ct (0.93, 0.78, 0.96)

Predicted promoter regions are underlined and transcription start points are in bold and capitalized. Putative TG-dinucleotide is indicated in bold italic and putative ribosome binding site are shown in gray boxes. The numbers in parentheses are predicted promoter scores.

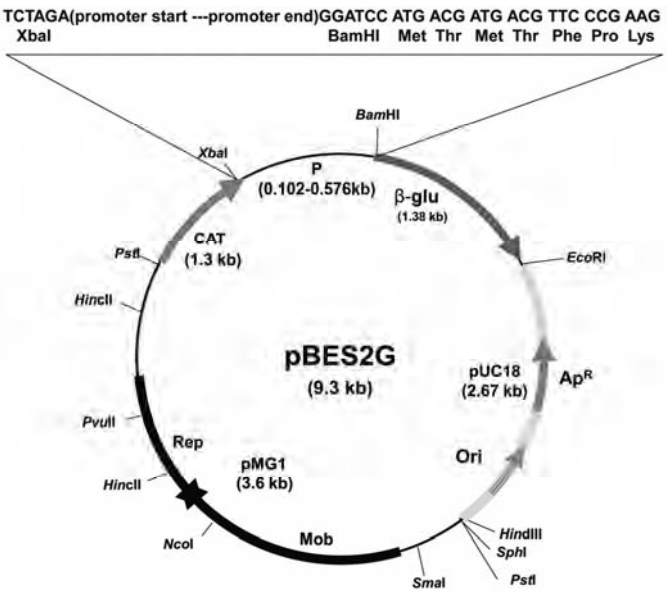

Fig. 1. Schematic map of the promoter screening vector pBES2G containing the *E. coli* origin of replication (Ori). The β-glucosidase reporter gene is in broken line, the chloramphenicol acetyl transferase gene (CAT) is light gray, and the *B. longum* replicon pMG1 that predicted replication proteins Rep is in black. The cloning sites at the start of the β-glucosidase reporter gene are expanded and annotated with restriction sites (*Xba*I and *Bam*HI) plus translation products above the plasmid.

834 were in between. They were predicted to be involved in ribosomal structure (644, 1527, 919, 888), translation (895), cell wall biogenesis (834), and putative inner membrane protein (632). Accordingly, we assumed that these 7 genes have their own promoters that do not need any induction.

Cloning and sequence analysis of the putative promoter genes

The putative promoter regions of the 7 genes were PCR

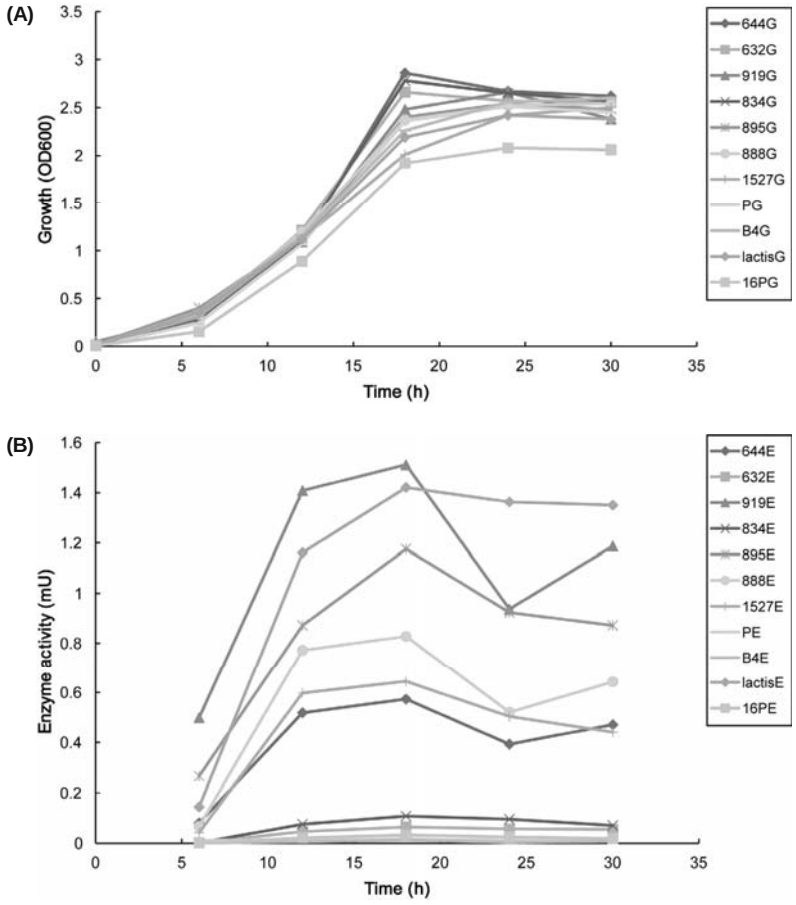

Fig. 2. Cell growth (A) and β-glucosidase activities (B) of recombinant *B. bifidum* BGN4 harboring each numbered promoter. In the legends, G and E mean growth (OD_{600}) and enzyme activity of each transformant, respectively. P means recombinant with pBES2G and 16P means recombinant with 16S rRNA promoter of *B. longum* MG1. B4 and lactis mean *B. bifidum* BGN4 and *B. lactis* AD011.

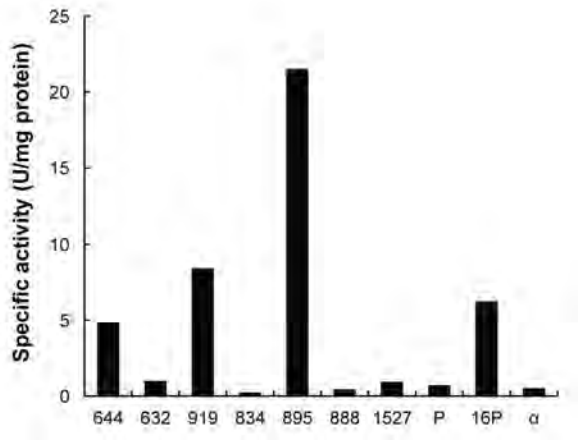

Fig. 3. Specific β-glucosidase activities of recombinant *E. coli* DH5α harboring various promoters grown in LB medium at 7 h. P, pBES2G; 16P, 16S rRNA promoter from *B. longum* MG1; α, *E. coli* DH5α

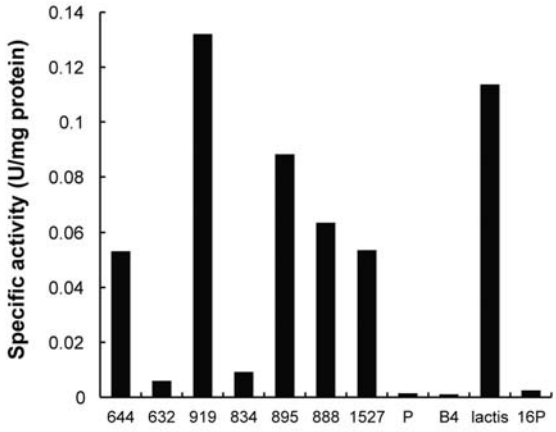

Fig. 4. Specific β-glucosidase activities of *B. lactis* AD011 and recombinant *B. bifidum* BGN4 with various promoters and in MRS at 18 h. The legends for each strain are the same as those of Fig. 2.

amplified and cloned as described in the 'Materials and Methods' section. The amplified fragments were 102–576 bp in size. The promoter prediction revealed one possible promoter region in P632, P834, P895, P919, P888, P1527, and three in P888. The -16 sequence and TG motif was observed (Table 4).

Construction of expression vectors using promoters

The β-glucosidase gene (YP_002469020 of CP001213) from *B. lactis* AD011 was cloned into *E. coli-Bifidobacterium* shuttle vector pBES2 to construct pBES2G (Fig. 1). β-Glucosidase was used as a reporter to evaluate each promoter's expression level. Each putative promoter region was inserted upstream of β-glucosidase gene in the pBES2G vector and transferred to *E. coli* DH5α (Fig. 1). The promoterless vector pBES2G was used as a negative control. Each recombinant expression vector was then purified from *E. coli* DH5α, methylated *in vitro*, and transformed into *B. bifidum* BGN4.

Cell growth and promoter activity assay

To examine the activity of each promoter, cells of all wild-type and recombinant strains were cultivated as described in the 'Materials and Methods' section. The promoter activities were analyzed by measuring β-glucosidase using *p*-nitrophenyl-β-glucopyranoside as a substrate. As expected, all promoters were shown to produce β-glucosidase when *B. bifidum* BGN4 was used as the expression host (Fig. 2), whereas promoterless vector and wild-type *B. bifidum* BGN4 did not show β-glucosidase activity (Fig. 2). The β-glucosidase activity increased with cell growth and decreased after the stationary phase. Maximal activity was observed at 18 h at the final stage of the exponential phase (Fig. 2).

The P919 promoter showed the strongest specific β-glucosidase activity among the promoters examined in *B. bifidum* BGN4 at 18 h, while the others were weaker than that of the original host *B. lactis* AD011.

The enzyme activities of recombinant *E. coli* DH5α were higher than their corresponding activities of recombinant *B. bifidum* BGN4 and the maximum activity was detected at 7 h (data not shown). Among them, P895 was the strongest in *E. coli* DH5α and P919 was the second. Recombination with P888, P834, P1527, or without promoter produced little activity as shown in wild-type *E. coli* DH5α (Fig. 3).

Discussion

Microarray analysis of total RNA from *B. bifidum* BGN4 revealed 7 genes showing relatively high-level expression among 1,000 genes during cultivation with or without pH control. However, these data represent promoter strength at the transcription level. The promoters should be analyzed at the translation level to determine their strength as well. For this reason, we established a promoter screening host-vector system using β-glucosidase as a reporter and β-glucosidase-negative *B. bifidum* BGN4 as a host. Seven putative promoters from the *B. bifidum* BGN4 were then cloned and their promoter activities were analyzed by the measurement of β-glucosidase activity in both *B. bifidum* BGN4 and *E. coli* DH5α. Based on these results, P919 and P895 yielded the strongest enzyme activities, while microarray showed that P644 and P1527 were stronger than P919 and P895 (Table 3). This result suggests that we have to consider the expression levels at both the translational and the transcriptional stages to screen strong promoters.

In our previous study, we constructed an expression vector using a 16S rRNA promoter from *B. longum* MG1 and successfully expressed cholesterol oxidase in both *E. coli* and *B. longum*. However, this promoter unexpectedly failed to produce β-glucosidase activity in *B. bifidum* BGN4 (Fig. 4), although it showed moderate enzyme activity in *E. coli* DH5α (Fig. 4). This might be explained by the absence of homology between the promoter region of the 16S rRNA of *B. bifidum* and that of *B. longum* (data not shown). This result indicates that each promoter has host specificity. These genes were predicted to encode ribosomal proteins (919, 895, 1527) and the peptide elongation factor Tu (644). Kim

et al. (2009) also cloned the tuf promoter as a strong promoter for *Lactococcus* according to microarray analysis of *Lactococcus lactis*.

Although one study reported that lactic acid bacteria promoters have the similar activities in *L. lactis* and *E. coli* (Jeong *et al.*, 2006), there is no such report for *Bifidobacterium*. In this study, the promoter activities in *E. coli* DH5α were higher than those in *B. bifidum* BGN4. This might be due to the host range of the respective promoters (Kim *et al.*, 2009). The copy number of the same shuttle vectors may affect their promoter activities when they are present in different hosts. The *E. coli–Bifidobacterium* shuttle vector pBES2 harbors *ori* for *E. coli* and *B. longum* MG1. The difference of the *Bifidobacterial* species can affect the activity of the promoter and *ori*. The growth rates of the recombinant *E. coli* DH5α at 7 h were also greater than those of recombinant *B. bifidum* BGN4 at 18 h.

The analysis of the activity patterns of the promoter in *B. bifidum* BGN4 suggested that promoter activity was growth-associated, revealing a general characteristic of constitutively expressed genes. There was no significant difference in growth patterns between *B. bifidum* BGN4 and recombinant *B. bifidum* BGN4 strains (Fig. 2). This result suggested that the strong promoters developed in this study did not inhibit the growth of *B. bifidum* BGN4.

Many studies have shown that the -10 and/or -35 hexamer sequences greatly affect promoter strength (McCracken *et al.*, 2000). Additionally, other factors such as TG motif (Helmann, 1995), spacers, UP elements (Estrem *et al.*, 1998), and promoters' 3-dimensional structures were reported to affect transcription efficiency (Jensen and Hammer, 1998). In many Gram-positive bacteria, the -16 region TG motif was observed 1 bp upstream of the -10 sequence (Voskuil and Chambliss, 1998). However, the -16 region TG motif was found -6 bp upstream of the -10 sequence in the 7 promoters of *B. bifidum* BGN4 (Table 4).

When the putative promoter sequences were analyzed using NCBI BlastN database, all of them showed homology only within other *B. bifidum* genomes except for P919. Interestingly, sequences that were highly homologous with P919 were found in *B. bifidum* (100%), *Bifidobacterium dentium* (90%), *B. longum* (95%), *Bifidobacterium adolescentis* (88%), and *Bifidobacterium animalis* (83%). Therefore, we suggest that promoter P919 may work in these *Bifidobacterium* species with strong activity. The presently characterized promoters will be useful for the development of food-grade expression systems for *Bifidobacterium*.

Acknowledgements

This research was supported by a grant (MG 08-0303-4-0) from the Microbial Genomics and Application Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science and Technology of the Republic of Korea and the Next-Generation BioGreen 21 Program (No.PJ008005), Rural Development Administration, Republic of Korea.

References

Bezkorovainy, A. and Miller-Catchpole, R. 1989. Biochemistry and physiology of Bifidobacteria. CRC Press. Boca Raton, Florida, USA.

Estrem, S.T., Gaal, T., Ross, W., and Gourse, R.L. 1998. Identification of an UP element consensus sequence for bacterial promoters. *Proc. Natl. Acad. Sci. USA* **95**, 9761–9766.

Gibson, J.L. and Tabita, F.R. 1996. The molecular regulation of the reductive pentose phosphate pathway in Proteobacteria and Cyanobacteria. *Arch. Microbiol.* **166**, 141–150.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580.

Helmann, J.D. 1995. Compilation and analysis of *Bacillus subtilis* sigma A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res.* **23**, 2351–2360.

Jensen, P.R. and Hammer, K. 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl. Environ. Microbiol.* **64**, 82–87.

Jeong, D.W., Choi, Y.C., Lee, J.M., Kim, J.H., Lee, J.H., Kim, K.H., and Lee, H.J. 2006. Isolation and characterization of promoters from *Lactococcus lactis* ssp. *cremoris* LM0230. *Food Microbiol.* **23**, 82–89.

Kim, E.B., Piao da, C., Son, J.S., and Choi, Y.J. 2009. Cloning and characterization of a novel tuf promoter from *Lactococcus lactis* subsp. *lactis* IL1403. *Curr. Microbiol.* **59**, 425–431.

Kim, J.Y., Wang, Y., Park, M.S., and Ji, G.E. 2010. Improvement of transformation efficiency through *in vitro* methylation and SacII site mutation of plasmid vector in *Bifidobacterium longum* MG1. *J. Microbiol. Biotechnol.* **20**, 1022–1026.

Klijn, A., Moine, D., Delley, M., Mercenier, A., Arigoni, F., and Pridmore, R.D. 2006. Construction of a reporter vector for the analysis of *Bifidobacterium longum* promoters. *Appl. Environ. Microbiol.* **72**, 7401–7405.

Kullen, M.J. and Klaenhammer, T.R. 2000. Genetic modification of intestinal lactobacilli and bifidobacteria. *Curr. Issues Mol. Biol.* **2**, 41–50.

McCracken, A., Turner, M.S., Giffard, P., Hafner, L.M., and Timms, P. 2000. Analysis of promoter sequences from *Lactobacillus* and *Lactococcus* and their activity in several *Lactobacillus* species. *Arch. Microbiol.* **173**, 383–389.

Mitsuoka, T. 1990. Bifidobacteria and their role in human health. *Indust. Microbiol.* **6**, 263–268.

Park, M.S., Kwon, B., Shim, J.J., Huh, C.S., and Ji, G.E. 2008. Heterologous expression of cholesterol oxidase in *Bifidobacterium longum* under the control of 16S rRNA gene promoter of bifidobacteria. *Biotechnol. Lett.* **30**, 165–172.

Park, M.S., Moon, H.W., and Ji, G.E. 2003. Molecular characterization of plasmid from *Bifidobacterium longum*. *J. Microbiol. Biotechnol.* **12**, 457–462.

Rist, M. and Kertesz, M.A. 1998. Construction of improved plasmid vectors for promoter characterization in *Pseudomonas aeruginosa* and other Gram-negative bacteria. *FEMS Microbiol. Lett.* **169**, 179–183.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA.

Sgorbati, B., Scardovi, V., and Leblanc, D.J. 1982. Plasmids in the genus *Bifidobacterium*. *J. Gen. Microbiol.* **128**, 2121–2131.

Sirard, J.C., Mock, M., and Fouet, A. 1995. Molecular tools for the study of transcriptional regulation in *Bacillus anthracis*. *Res. Microbiol.* **146**, 729–737.

Voskuil, M.I. and Chambliss, G.H. 1998. The -16 region of *Bacillus subtilis* and other Gram-positive bacterial promoters. *Nucleic Acids Res.* **26**, 3584–3590.

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i12.1308

World J Gastroenterol 2012 March 28; 18(12): 1308-1318
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Probiotic modulation of dendritic cells co-cultured with intestinal epithelial cells

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Author contributions: Kim JY performed most of the experiments; Park MS prepared and provided the probiotic bacteria and was involved in editing the manuscript; Kim JY and Ji GE designed the study and wrote the manuscript; Ji GE acted as a principle investigator.
Supported by The Small and Medium Business Administration, No. S1072365; and the Next-Generation BioGreen 21 Program, No. PJ008005, Rural Development Administration, South Korea
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Received: June 9, 2011 Revised: September 30, 2011
Accepted: January 22, 2012
Published online: March 28, 2012

Abstract

AIM: To investigate cytokine production and cell surface phenotypes of dendritic cells (DC) in the presence of epithelial cells stimulated by probiotics.

METHODS: Mouse DC were cultured alone or together with mouse epithelial cell monolayers in normal or inverted systems and were stimulated with heat-killed probiotic bacteria, *Bifidobacterium lactis* AD011 (BL), *Bifidobacterium bifidum* BGN4 (BB), *Lactobacillus casei* IBS041 (LC), and *Lactobacillus acidophilus* AD031 (LA), for 12 h. Cytokine levels in the culture supernatants were determined by enzyme-linked immunosorbent assay and phenotypic analysis of DC was investigated by flow cytometry.

RESULTS: BB and LC in single-cultured DC increased the expression of I-Ad, CD86 and CD40 (I-Ad, 18.51 *vs* 30.88, 46.11; CD86, 62.74 *vs* 92.7, 104.12; CD40, 0.67 *vs* 6.39, 3.37, $P < 0.05$). All of the experimental probiotics increased the production of inflammatory cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)-α. However, in the normal co-culture systems, LC and LA decreased the expression of I-A^d (39.46 *vs* 30.32, 33.26, $P < 0.05$), and none of the experimental probiotics increased the levels of IL-6 or TNF-α. In the inverted co-culture systems, LC decreased the expression of CD40 (1.36 *vs* -2.27, $P < 0.05$), and all of the experimental probiotics decreased the levels of IL-6. In addition, BL increased the production of IL-10 (103.8 *vs* 166.0, $P < 0.05$) and LC and LA increased transforming growth factor-β secretion (235.9 *vs* 618.9, 607.6, $P < 0.05$).

CONCLUSION: These results suggest that specific probiotic strains exert differential immune modulation mediated by the interaction of dendritic cells and epithelial cells in the homeostasis of gastrointestinal tract.

Key words: Dendritic cells; Intestinal epithelial cells; Probiotics; Co-culture; Immune modulation

Peer reviewers: Jianyuan Chai, PhD, MS, BS, Assistant Professor, Research (09-151), VA Long Beach Healthcare System, 5901 E. 7th St, Long Beach, CA 90822, United States; Emiko Mizoguchi, MD, PhD, Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States; Alain L Servin, PhD, Faculty of Pharmacy, French National Institute of Health and Medical Research, Unit 756, Rue J.-B. Clément, F-922296 Châtenay-Malabry, France

Kim JY, Park MS, Ji GE. Probiotic modulation of dendritic cells co-cultured with intestinal epithelial cells. *World J Gastroenterol* 2012; 18(12): 1308-1318 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i12/1308.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i12.1308

INTRODUCTION

The gastrointestinal (GI) tract is an immunologic organ

with continuous antigen exposure in the form of food, normal bacteria and pathogens. Despite numerous antigenic challenges, the complicated mucosal immune system maintains GI homeostasis *via* the concerted actions of the various mucosal immune cells. Dendritic cells (DC), dedicated antigen-presenting cells, modulate the immune balance in the GI tract[1]. DC can take up antigens directly by extending their dendrites into the lumen or indirectly after transport of the antigens by M cells overlying Peyer's patch[2,3]. Antigen-carrying DC may traffic through the lymphatics to the mesenteric lymph nodes[4], mediating the homing of activated effector/memory T cells and IgA-secreting B cells[5,6] and inducing regulatory T cells to produce interleukin (IL)-10 and transforming growth factor (TGF)-β[7,8]. These roles depend on the regulation of cell surface expression of co-stimulatory molecules and production of inflammatory chemokines and cytokines[9-11].

DC can recognize and present microbial components using pattern receptor system which includes toll-like-receptor (TLR). TLR can interact with microorganism-associated molecules such as peptidoglycan, lipoprotein, and lipopolysaccharide[12-16]. *Bifidobacterium* and *Lactobacillus* are major components of the commensal microbes of the GI tract and are frequently used as probiotics[17,18]. Probiotics, defined as live microorganisms which, when consumed in appropriate amounts in food, confer a health benefit on the host[19], exert various host physiological responses such as immunomodulatory effect[20]. Recent experiments reported that DC could be modulated by probiotics. Several *Lactobacillus* species could regulate DC surface expression and cytokine production[21]. In addition, the probiotics mixture VSL No. 3 upregulated the expression of major histocompatibility complex (MHC) class Ⅱ and co-stimulation molecules[22].

DC are often located close to epithelial cells, populating the subepithelial dome of Peyer's patches, immediately adjacent to the follicle-associated epithelium and the lamina propria[23,24]. Intestinal epithelial cells secrete many mediators, including functional peptides such as defensins, mucins, chemokines, and cytokines such as IL 8[25-27]. TLR5 on the epithelium is a key mediator of pro-inflammatory responses to flagella from commensal bacteria[28,29]. Flagella also stimulate the maturation of responsive DC[30].

Interaction between DC and epithelial cells is integral to the intestinal immune system. We hypothesized that epithelial cells stimulated by probiotics could regulate the maturation of DC. Accordingly, the present study investigated the pattern of cytokine production and the surface phenotype of DC in the presence of epithelial cells polarized by heat-killed probiotic bacteria.

MATERIALS AND METHODS

Preparation of probiotic bacteria

Bifidobacterium bifidum BGN4 (BB) was isolated from healthy infant fecal matter and identified in our laboratory[31]. *Bifidobacterium lactis* AD011 (BL), *Lactobacillus casei* IBS041 (LC), and *Lactobacillus acidophilus* AD031 (LA) were provided by the Research Institute of Bifido Co. Ltd. (Hongchun, Gangwondo, South Korea). Four probiotic bacteria were anaerobically propagated in de Man, Rogosa, and Sharpe (Difco, Detroit, MI, United States) broth containing 0.05% L-cysteine (Sigma, St. Louis, MO, United States) at 37 ℃ until mid-log phase was reached. Subsequently, probiotics were inoculated at 1% and anaerobically cultured in de Man, Rogosa, and Sharpe (Difco) broth containing 0.05% L-cysteine (Sigma) at 37 ℃. *Lactobacillus* species were incubated for 16 h, and *Bifidobacteirum* species were incubated for 24 h to late log phase. The bacteria were collected by centrifugation at 1000 × *g* for 15 min at 4 ℃ and washed twice with phosphate-buffered saline (PBS). After washing, the bacteria were resuspended in 1 mL of PBS and incubated at 95 ℃ for 30 min to prepare heat-killed bacteria cells. The killed bacteria were collected by centrifugation at 1000 × *g* for 15 min and then lyophilized (Combi-514R, Hanil Science Industrial, Seoul, South Korea).

Generation of CMT-93 monolayers

CMT93 was derived from carcinomas of C57BL mouse large intestine. The cells have an epithelial morphology and forms acini, junctional complexes, and microvilli with attached glycoprotein[32]. CMT-93 cells were maintained in DMEM (Gibco Life Technologies, United Kingdom) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, United Kingdom) and 1% penicillin/streptomycin (Invitrogen), and were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. Monolayers were grown in 24-well Corning Costar Transwell plates (Corning Inc., United States) with 3 μm pore-size filter inserts. In the normal co-culture system, 5×10^5 cells were seeded into the inserts, and the wells were filled with 1 mL medium. In the inverted co-culture system, inserts were removed and inverted in tissue culture dishes, and the cells of the same volume were seeded to the exposed filter membrane. The culture dishes were filled with enough medium to sink the inserts. The transwell inserts were cultured for 3-4 d until CMT-93 established monolayers. Confluence of the cells was confirmed when the trans-epithelial electrical resistance (TEER; Millicell ERS Ohmmeter, Millipore, Eschborn, Germany) exceeded the cut-off point of 250 Ω/cm^2.

JAWS Ⅱ cell preparation

JAWS Ⅱ, mouse bone marrow-derived immature DC[33], were maintained in α-MEM (Gibco) supplemented with 5 ng/mL GM-CSF (Sigma, St. Louis, MO, United States), 20% heat-inactivated FBS (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). The mixture was incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. The cells were cultured at a 1/2 subcultivation ratio for 5-6 d in complete medium.

Co-culture experiment model

The co-culture experiment model is shown in Figure 1. JAWS Ⅱ cells were harvested, washed, and resuspended in RPMI1640 complete medium (Gibco) containing 5 ng/mL GM-CSF (Sigma), 10% heat-inactivated FBS

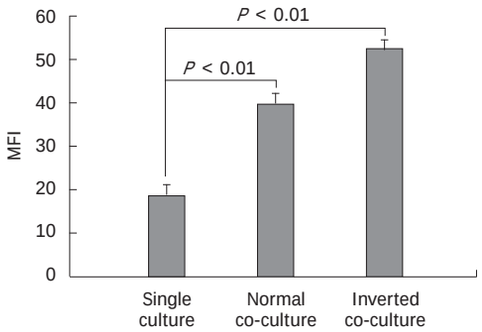


Figure 1 Effect of non-stimulated intestinal epithelial cells on surface phenotype of dendritic cells. Fluorescence activated cell sorter analysis of dendritic cells (DC) cultured alone or co-cultured with non-stimulated epithelial cell monolayers for 12 h showing DC surface phenotype by staining with I-A^d. Data are shown as the mean fluorescent intensity (MFI) ± SEM of three representative experiments. Significant difference between the single culture and co-culture as determined by Student's *t*-test ($P < 0.01$).

(Invitrogen), and 1% penicillin/streptomycin (Invitrogen). A total of 1×10^6 JAWS Ⅱ cells were added into lower chambers, and the normal and inverted cultured CMT-93 monolayer inserts were placed in the JAWS Ⅱ seeded transwell plates. One hundred μg/mL of the experimental bacteria or 10 μg/mL of LPS (Sigma) were added to the CMT-93 monolayer inserts. For comparison, JAWS Ⅱ cells were also plated at the same concentration in 24 well tissue culture plates (Corning Inc.), and the same amount of the bacteria or LPS were added to the cells. The single- or co-cultured cells were incubated with 1 mL RPMI1640 complete medium at 37 ℃ in a humidified atmosphere of 5% CO_2 for 12 h.

Flow cytometry analysis

Incubated JAWS Ⅱ cells were harvested and washed three times in cold FACS buffer (Dulbecco's PBS; Gibco, 2% FBS) and then stained with the appropriate monoclonal antibodies: PE-conjugated anti-I-A^d, anti-CD80, anti-CD86, and anti-CD40 at a final concentration of 10 μg/mL for 30 min at 4 ℃ in the dark. Isotype control antibodies were hamster IgG2 k, rat IgG2a k, and mouse IgG2b. The stained cells were analyzed immediately by FACSCalibur (Becton Dickinson, San Diego, CA, United States). All of the antibodies used in this flow cytometry analysis were purchased from Pharmingen (San Diego, CA, United States).

Cytokine measurement

JAWS Ⅱ cell supernatants were harvested from the lower chamber of the Transwell or from the JAWS Ⅱ cultured-alone plate following incubation, and were assayed for levels of IL-6, IL-10, IL-12p70, tumor necrosis factor (TNF)-α and TGF-β using enzyme-linked immunosorbent assay . Briefly, Nunc-Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 2 μg/mL of rat anti-mouse IL-6 and TGF-β capture antibodies in coating buffer (1.6 g/L Na_2CO_3, 7.1 g/L $NaHCO_3$), pH 9.5, or 2 μg/mL of rat anti-mouse IL-10, IL-12p70, and TNF-α capture antibodies in coating buffer (11.8 g/L Na_2HPO_4, 16.1 g/L NaH_2PO_4), pH 6.5, overnight at 4 ℃. After washing and blocking, 100 μL of 1:100 diluted (IL-6) or undiluted (IL-10, IL-12p70, TNF-α and TGF-β) supernatant was added to individual wells and incubated overnight at 4 ℃. Plates were washed, and biotinylated rat anti-mouse IL-6, IL-10, IL-12p70, TNF-α and TGF-β monoclonal antibodies (2 μg/mL) and HRP-conjugated streptavidin were added to the plates for cytokine detection for 1 h at room temperature. The reactions were developed with the 3,3',5,5'-tetramethylbenzidine substrate (Fluka, Neu-Ulm, Switzerland) for 30 min at room temperature. The color reactions were stopped with 2 N H_2SO_4 and analyzed at 450 nm. Equivalent levels of IL-6, IL-10, IL-12p70, TNF-α and TGF-β were measured for comparison with a reference curve generated using standards of these cytokines.

Statistical analyses

Data are presented as the mean ± SE, indicated by bars in the figures. All statistical analyses were performed using SPSS 12.0K for Windows (SPSS Inc., Chicago, IL, United States). Differences between the single culture and co-culture were determined by Student's t-test, and differences between cytokine levels were analyzed by analysis of variance followed by Duncan's multiple range test. The *P* values < 0.05 were considered to be statistically significant.

RESULTS

Development of stable CMT-93 epithelial cell monolayers

To obtain stable CMT-93 intestinal epithelial cell monolayers, we monitored the culture every day for TEER using a Millicell-ERS ohmmeter for a period of 7 d. On day 3, normal insert monolayer integrity was obtained at 300-500 Ω/cm^2, and inverted insert monolayer integrity was obtained at 250-350 Ω/cm^2. In addition, the generation of epithelial cell monolayers was observed on the surface of the inserts by microscope (data not shown). Monolayers between day 3 and 4 were used for co-culture experiments. After co-culture with DC for 12, the integrity of CMT-93 monolayer was evaluated by TEER. There was no difference between before and after co-culture in terms of the resistances within the margin of error.

Dendritic cells phenotype modulation during co-culturing with epithelial cells

DC surface phenotypes were compared in the presence and absence of epithelial cells. The expression of MHC class Ⅱ I-A^d on the normal and the inverted co-cultured DCs was upregulated compared with that of the single-cultured DC (single culture, 18.51 ± 2.86; normal co-culture, 39.46 ± 2.53; inverted co-culture, 52.03 ± 2.41; Figure 1). Co-culture with epithelial cells did not alter the DC surface expression of CD80, CD86 and CD40 (data not shown).

Effect of probiotics on the expression of major histocompatibility complex class Ⅱ and costimulatory molecules

We performed flow cytometery analyses to examine the

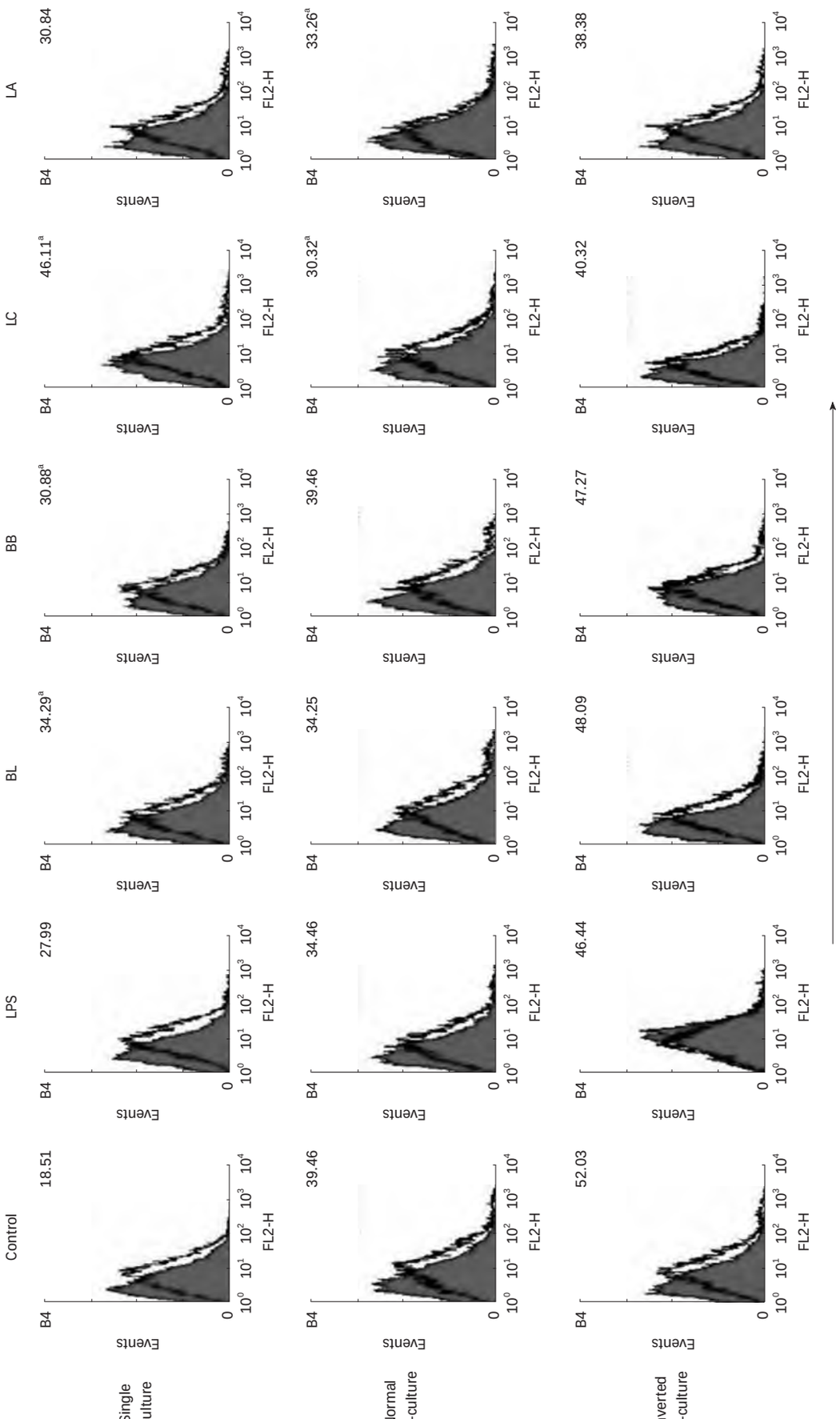
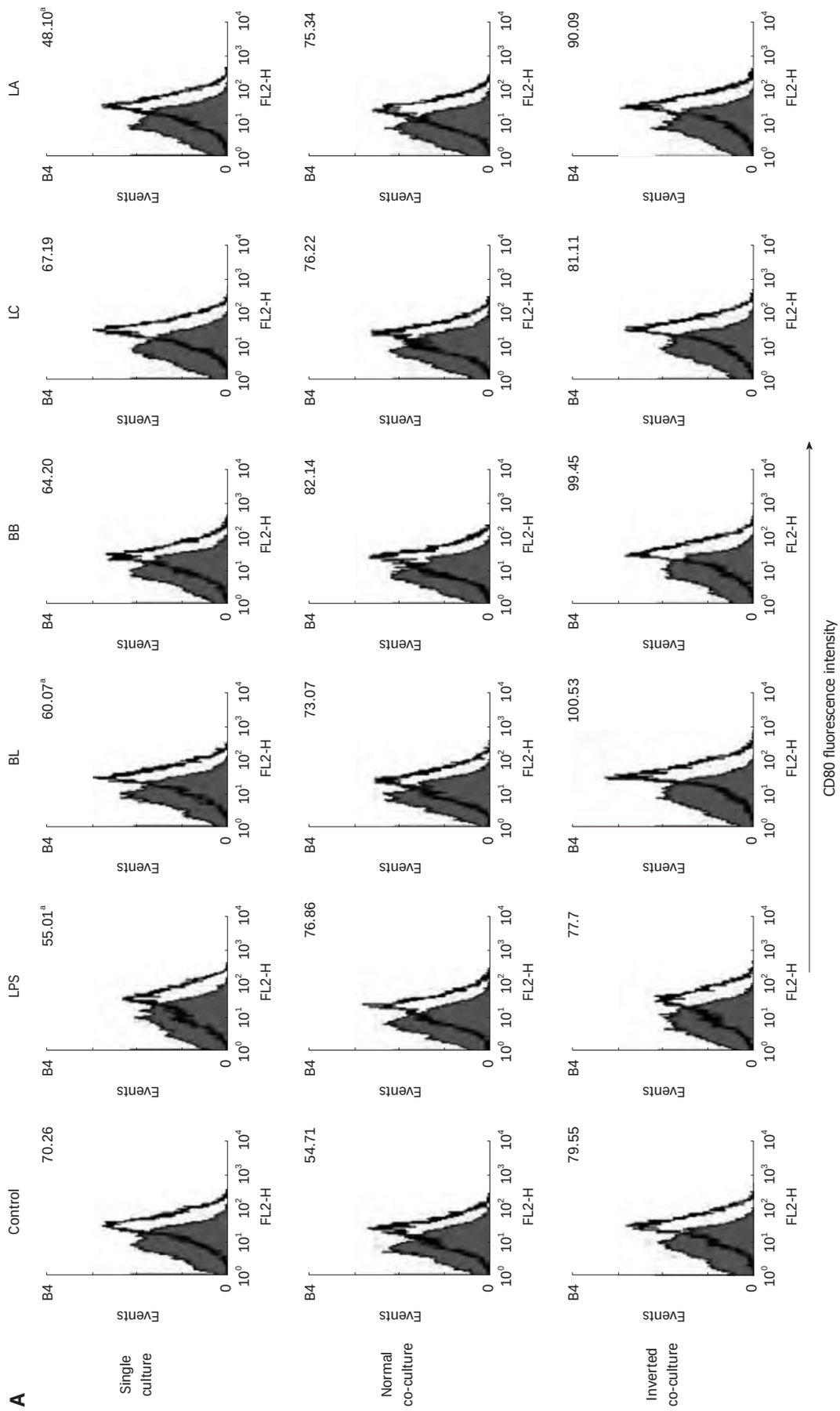


Figure 2 Effect of probiotics on I-A^d of single- or co-cultured dendritic cells. Fluorescence activated cell sorter analysis of probiotics-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Filled histograms are isotype controls; unfilled histogram show staining for I-A^d. Numbers indicate the mean fluorescent intensity of three representative experiments. [a]Significant difference among the control, lipopolysaccharides and probiotics as determined by analysis of variance ($P < 0.05$). LPS: Lipopolysaccharides; BL: *Bifidobacterium lactis* AD011, BB: *Bifidobacterium bifidum* BGN4, LC: Lactobacillus casei IBS041; LA: Lactobacillus acidophilus AD031.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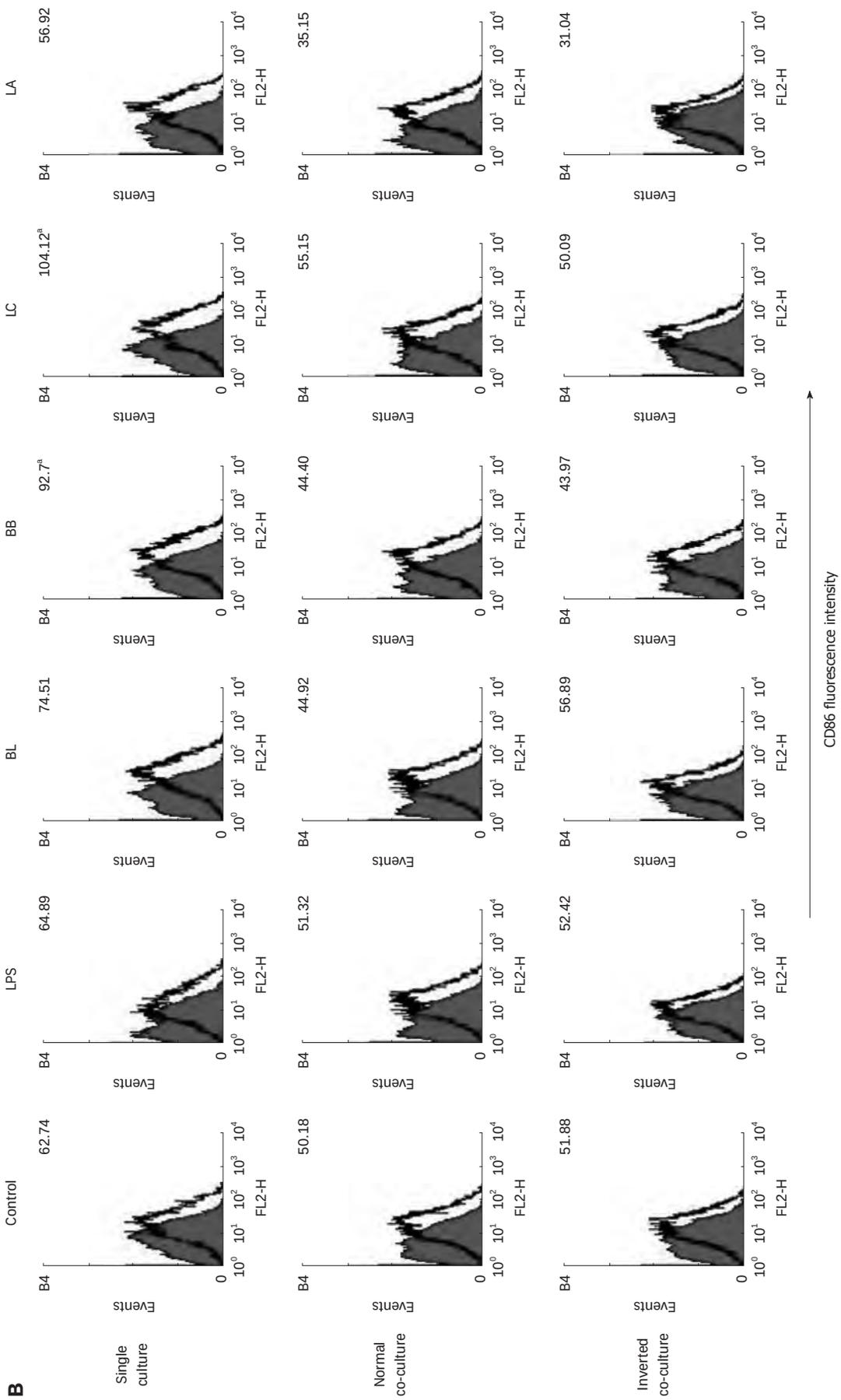
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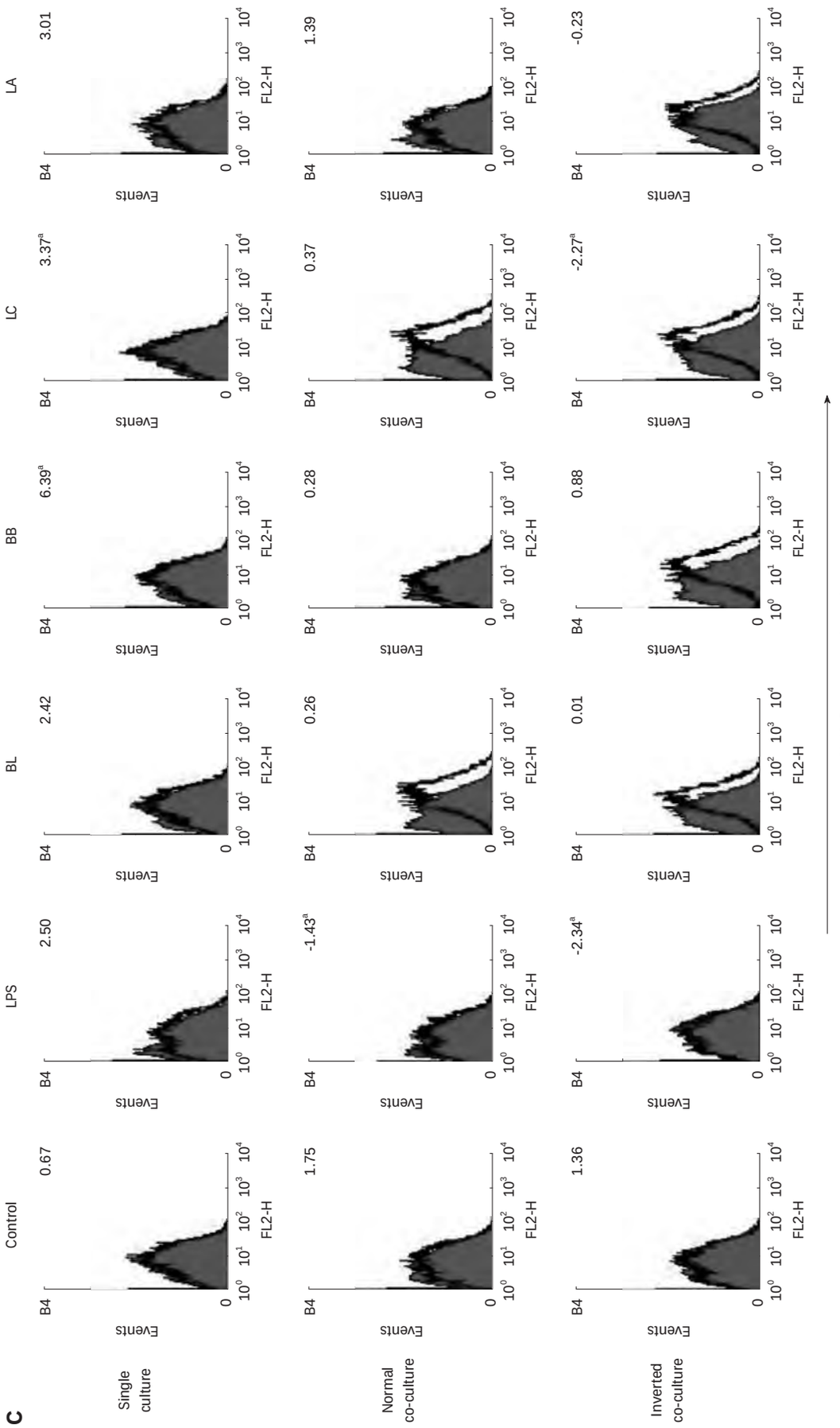

Figure 3 Effect of probiotics on the CD80, CD86 and CD40 of single- or co-cultured dendritic cells. Fluorescence activated cell sorter analysis of probiotics-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Filled histograms are isotype controls; unfilled histogram shows staining for CD80 (A), CD86 (B) and CD40 (C). Numbers indicate the mean fluorescent intensity of at least three representative experiments. [a]Significant difference among the control, lipopolysaccharides, and probiotics as determined by analysis of variance ($P < 0.05$). LPS: Lipopolysaccharides; BL: *Bifidobacterium lactis* AD011, BB: *Bifidobacterium bifidum* BGN4, LC: Lactobacillus casei IBS041; LA: Lactobacillus acidophilus AD031.

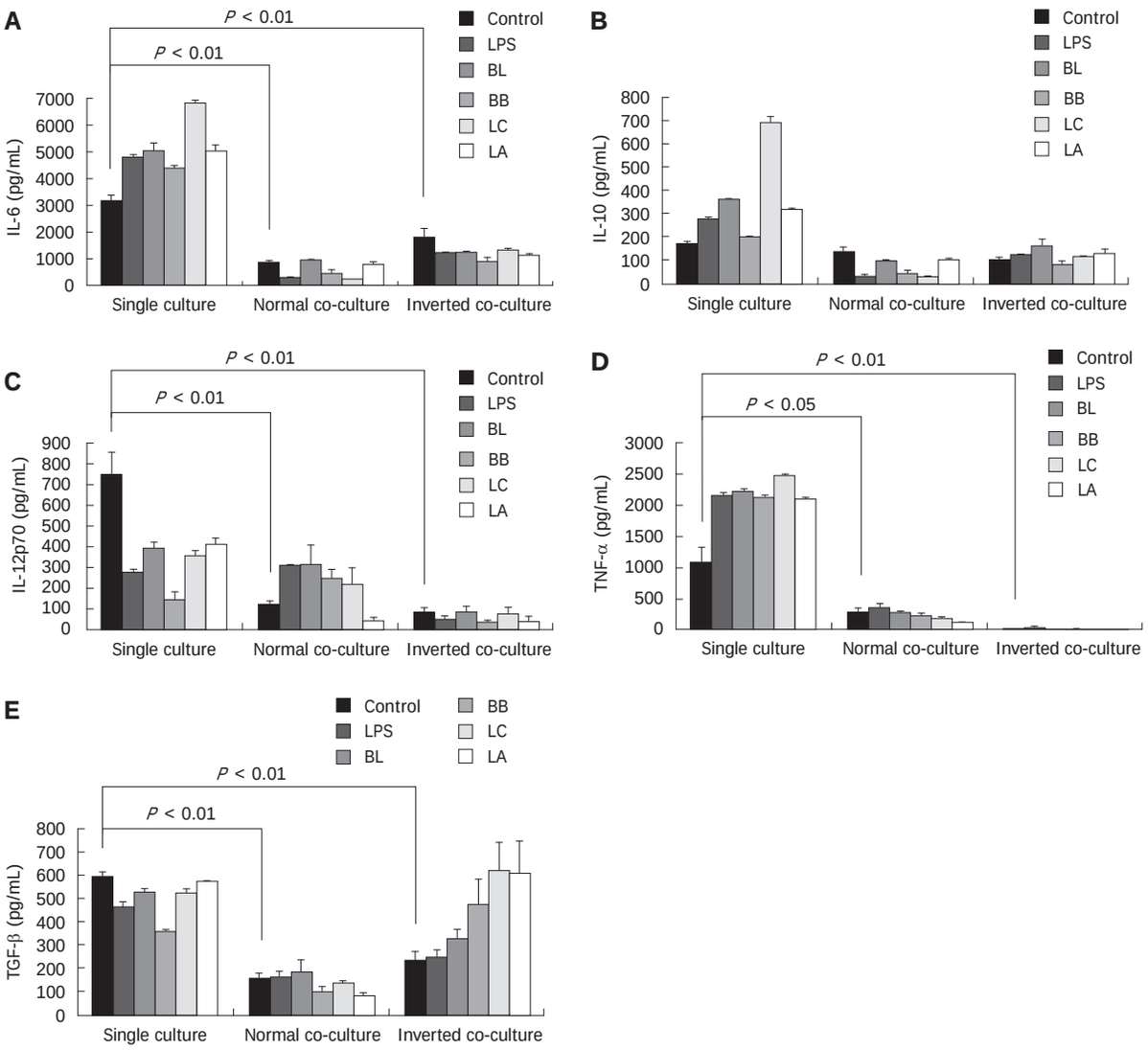

Figure 4 Effect of probiotics on the production of cytokines from single- or co-cultured dendritic cells. Supernatants were obtained from probiotic-treated dendritic cells (DC) cultured in the presence or absence of intestinal monolayers for 12 h. Levels of interleutkin (IL)-6 (A), IL-10 (B), IL-12p70 (C), tumor necrosis factor (TNF)-α (D), and transforming growth factor(TGF)-β (E) were determined by enzyme-linked immunosorbent assay. Data are shown as mean ± SE of three representative experiments. Different letters indicate significant differences among the control, lipopolysaccharides (LPS), and probiotics determined by Duncan's multiple range test ($P < 0.05$). Significant difference between the single culture and co-culture as determined by Student's *t*-test ($P < 0.05$). BL: *Bifidobacterium lactis* AD011; BB: *Bifidobacterium bifidum* BGN4; LC: *Lactobacillus casei* IBS041; LA: *Lactobacillus acidophilus* AD031.

12p70 secretion. IL-12 directed the differentiation of T cells to a Th1 phenotype[41]. Nier reported that *Bifidobacterium bifidum* enhanced the expression of CD86 and MHC class Ⅱ in human neonatal DC, which led in turn to the polarization of IFN-γ-producing T cells[42]. Mohamadzadeh *et al*[43] showed that *Lactobacillus gasseri, Lactobacillus johnsonni*, and *Lactobacillus reuteri* upregulated the expression of MHC class Ⅱ, CD40, CD80 and CD86 in human myeloid DC and increased the level of IL-12p70 which induced the polarization from CD4(+) and CD8(+) T cells to T helper 1 and Tc1 cells. Meanwhile, Drakes *et al*[22] showed that probiotic products containing *Lactobacillus* and *Bifidobacterium* upregulated the expression of MHC class Ⅱ, CD40, CD80 and CD86, and did not induce the production of IL-12p70 in mouse DC. Additionally, mouse bone marrow-derived DC treated with *Lactobacillus reuteri* induced Th2 immune response[21]. Taken together, the results of these earlier studies suggested that probiotics upregulated the expression of MHC class Ⅱ and differently modulated co-stimulatory molecules such as IL-12p70 and T cell polarization, depending on the DC origin and the strain of probiotics.

Interestingly, in the present study the effects of probiotics on cytokine production and the surface phenotype in co-cultured DC with epithelial cells were markedly different from those in single-cultured DC. All of the experimental probiotics induced the production of pro-inflammatory cytokines, IL-6 and TNF-α, in the single cultured DC. TNF-α mediated various immune responses[44], and over-production of TNF-α could play a role in tissue damage and intestinal pathologies[45,46]. In contrast with the results from the single system the experimental probiotics reduced or did not affect the expression of I-A^d, CD86 and CD40 or the production of IL-6, IL-12p70

19 **Food and Agriculture Organization**, World Health Organization. 2001. Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Cordoba, Argentina 1 to 4 October 2001. **Available** from: URL: **http://www.who.int/foodsafety/publications/** fs_management/en/probiotics.pdf

20 **Salminen S**, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I. Functional food science and gastrointestinal physiology and function. *Br J Nutr* 1998; **80** Suppl 1: S147-S171

21 **Thomas MJ**, Noble A, Sawicka E, Askenase PW, Kemeny DM. CD8 T cells inhibit IgE via dendritic cell IL-12 induction that promotes Th1 T cell counter-regulation. *J Immunol* 2002; **168**: 216-223

22 **Drakes M**, Blanchard T, Czinn S. Bacterial probiotic modulation of dendritic cells. *Infect Immun* 2004; **72**: 3299-3309

23 **Iwasaki A**, Kelsall BL. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *J Exp Med* 2000; **191**: 1381-1394

24 **Bell SJ**, Rigby R, English N, Mann SD, Knight SC, Kamm MA, Stagg AJ. **Migration and maturation of human colonic** dendritic cells. *J Immunol* 2001; **166**: 4958-4967

25 **Corfield AP**, Myerscough N, Longman R, Sylvester P, Arul S, Pignatelli M. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut* 2000; **47**: 589-594

26 **Takahashi A**, Wada A, Ogushi K, Maeda K, Kawahara T, Mawatari K, Kurazono H, Moss J, Hirayama T, Nakaya Y. Production of beta-defensin-2 by human colonic epithelial cells induced by Salmonella enteritidis flagella filament structural protein. *FEBS Lett* 2001; **508**: 484-488

27 **Stadnyk AW**. Intestinal epithelial cells as a source of inflammatory cytokines and chemokines. *Can J Gastroenterol* 2002; **16**: 241-246

28 **Tallant T**, Deb A, Kar N, Lupica J, de Veer MJ, DiDonato JA. Flagellin acting via TLR5 is the major activator of key signaling pathways leading to NF-kappa B and proinflammatory gene program activation in intestinal epithelial cells. *BMC Microbiol* 2004; **4**: 33

29 **Bambou JC**, Giraud A, Menard S, Begue B, Rakotobe S, Heyman M, Taddei F, Cerf-Bensussan N, Gaboriau-Routhiau V. In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal Escherichia coli strain. *J Biol Chem* 2004; **279**: 42984-42992

30 **Equils O**, Schito ML, Karahashi H, Madak Z, Yarali A, Michelsen KS, Sher A, Arditi M. Toll-like receptor 2 (TLR2) and TLR9 signaling results in HIV-long terminal repeat transactivation and HIV replication in HIV-1 transgenic mouse spleen cells: implications of simultaneous activation of TLRs on HIV replication. *J Immunol* 2003; **170**: 5159-5164

31 **Park SY**, Ji GE, Ko YT, Jung HK, Ustunol Z, Pestka JJ. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of Bifidobacterium. *Int J Food Microbiol* 1999; **46**: 231-241

32 **Franks LM**, Hemmings VJ. A cell line from an induced carcinoma of mouse rectum. *J Pathol* 1978; **124**: 35-38

33 **Jiang X**, Shen C, Rey-Ladino J, Yu H, Brunham RC. Characterization of murine dendritic cell line JAWS II and primary bone marrow-derived dendritic cells in Chlamydia muridarum antigen presentation and induction of protective immunity. *Infect Immun* 2008; **76**: 2392-2401

34 **Corthésy B**, Gaskins HR, Mercenier A. Cross-talk between probiotic bacteria and the host immune system. *J Nutr* 2007; **137**: 781S-790S

35 **Kim HY,** Ji GE. **Effect of viability and integrity of Bifidobacterium on suppression of allergy in mice.** *J Microbiol Biotechnol* 2006; **16:** 1010-1016

36 **Tikhonova GP**, Bizin IuP. [Pathomorphologic changes in the organs of rats following chronic inhalation exposure to liquid polyethylsiloxane]. *Kosm Biol Aviakosm Med* **1976; 10**: 69-74

37 **Huang FP**, Platt N, Wykes M, Major JR, Powell TJ, Jenkins CD, MacPherson GG. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med* 2000; **191**: 435-444

38 **Niess JH**, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 2005; **307**: 254-258

39 **Pulendran B**, Kumar P, Cutler CW, Mohamadzadeh M, Van Dyke T, Banchereau J. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *J Immunol* 2001; **167**: 5067-5076

40 **Sato A**, Iwasaki A. Peyer's patch dendritic cells as regulators of mucosal adaptive immunity. *Cell Mol Life Sci* 2005; **62**: 1333-1338

41 **Hsieh CS**, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 1993; **260**: 547-549

42 **Niers LE**, Hoekstra MO, Timmerman HM, van Uden NO, de Graaf PM, Smits HH, Kimpen JL, Rijkers GT. Selection of probiotic bacteria for prevention of allergic diseases: immunomodulation of neonatal dendritic cells. *Clin Exp Immunol* 2007; **149**: 344-352

43 **Mohamadzadeh M**, Olson S, Kalina WV, Ruthel G, Demmin GL, Warfield KL, Bavari S, Klaenhammer TR. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc Natl Acad Sci U S A* 2005; **102**: 2880-2885

44 **Vassalli P**. The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 1992; **10**: 411-452

45 **Graves DT**. The potential role of chemokines and inflammatory cytokines in periodontal disease progression. *Clin Infect Dis* 1999; **28**: 482-490

46 **Baert FJ**, D'Haens GR, Peeters M, Hiele MI, Schaible TF, Shealy D, Geboes K, Rutgeerts PJ. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. *Gastroenterology* 1999; **116**: 22-28

47 **Haller D,** Bode C, Hammes WP, Pfeifer AMA, Schiffrin EJ, Blum S, Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut* 2000; **47:** 79-87

48 **Smits HH**, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, Zaat BA, Yazdanbakhsh M, Wierenga EA, van Kooyk Y, Kapsenberg ML. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* 2005; **115**: 1260-1267

49 **Weiner HL**. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 2001; **182**: 207-214

50 **Weiner HL**. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes Infect* 2001; **3**: 947-954

51 **Mowat AM**, Donachie AM, Parker LA, Robson NC, Beacock-Sharp H, McIntyre LJ, Millington O, Chirdo F. The role of dendritic cells in regulating mucosal immunity and tolerance. *Novartis Found Symp* 2003; **252**: 291-302; discussion 302-305

S- Editor Shi ZF **L- Editor** O'Neill M **E- Editor** Xiong L

J. Microbiol. Biotechnol. (2012), **22**(12), 1714–1723
http://dx.doi.org/10.4014/jmb.1208.08028
First published online September 21, 2012
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Identification of the β-Glucosidase Gene from *Bifidobacterium animalis* subsp. *lactis* and Its Expression in *B. bifidum* BGN4

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Received: August 14, 2012 / Revised: September 5, 2012 / Accepted: September 9, 2012

β-Glucosidase is necessary for the bioconversion of glycosidic phytochemicals in food. Two *Bifidobacterium* strains (*Bifidobacterium animalis* subsp. *lactis* SH5 and *B. animalis* subsp. *lactis* RD68*)* with relatively high β-glucosidase activities were selected among 46 lactic acid bacteria. A β-glucosidase gene (*bbg572*) from *B. lactis* was shotgun cloned, fully sequenced, and analyzed for its transcription start site, structural gene, and deduced transcriptional terminator. The structural gene of *bbg572* was 1,383 bp. Based on amino sequence similarities, *bbg572* was assigned to family 1 of the glycosyl hydrolases. To overexpress *bbg572* in *Bifidobacterium*, several bifidobacteria expression vectors were constructed by combining several promoters and a terminator sequence from different bifidobacteria. The maximum activity of recombinant Bbg572 was achieved when it was expressed under its own promoter and terminator. Its enzyme activity increased 31-fold compared with those of its parental strains. The optimal pH for Bbg572 was pH 6.0. Bbg572 was stable at 37–40°C. It hydrolyzed isoflavones, quercetins, and disaccharides with various β-glucoside linkages. Bbg572 also converted the ginsenosides Rb1 and Rb2. These results suggest that this new β-glucosidase-positive *Bifidobacterium* transformant can be utilized for the production of specific aglycone products.

Keywords: β-Glucosidase, *Bifidobacterium*, promoter, terminator

β-Glucosidase is an important enzyme that hydrolyzes β-glucosides by cleaving the β-D-glucosidic linkages and liberating glucose moieties [8]. β-Glucosidases are widely distributed in living organisms and play vital roles in many biological processes, such as the degradation of cellulosic biomass [11], cyanogenesis [24], the cleavage of glucosylated flavonoids [25], and the production of fuel ethanol from lignocelluloses [23]. A number of biologically active components of natural products, such as ginsenoside, isoquercetin, daidzein, and genistein, are glycosides that are important functional ingredients of natural food materials. Interestingly, the biological effects of many glycosides are not attributable to their glycoside forms, but to their aglycones [15, 19]. Aglycones are highly bioactive because of their unimpeded intestinal absorption, unlike the corresponding glycosides, which are not absorbed across enterocytes because of their greater hydrophilicity and higher molecular weights [3, 34, 38]. When glycosides are ingested as food components, their bioavailability can be enhanced by the hydrolysis of their sugar moieties by the various glycosidases of the intestinal microflora. Intestinal microflora differ between individuals, so the bioavailabilities of the glycosidic phytochemicals differ. To overcome these individual differences in the bioavailability of phytochemicals in food materials, the bioconversion of phytochemicals by glycosidases before oral administration has been studied.

The genus *Bifidobacterium* is recognized by the World Health Organization and the Food and Agriculture Organization as one of the most important probiotic microorganisms [18], except for *B. scardovii* and *B. dentium*, which might cause clinical infection and dental caries [7, 36]. Several groups of bacteria are known to express β-glucosidase activity, including species of *Bifidobacterium* [13], which are a major component of the human gastrointestinal tract microflora. Previously, β-glucosidase was expressed in *Escherichia coli* [20, 27–29] and used in various fields related to carbohydrate chemistry. However, safety problems must be addressed before recombinant enzymes are used in the food industry. Because it is generally nonpathogenic, *Bifidobacterium* may be advantageous as the host cells to produce various recombinant food-grade enzymes.

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For this purpose, Kim *et al.* [22] cloned the structural gene of β-glucosidase from *B. lactis* AD011 at the downstream of the 16S rRNA promoter and ribosome binding site (RBS) of the bifiobacterial expression vector, pBES16PR, and expressed in *E. coli*. However, they did not show any detectable β-glucosidase activity in *B. bifidum* BGN4.

To overcome our previous study, we have cloned, sequenced, and sucessfully overexpressed the bifidobacterial β-glucosidase gene in *B. bifidum* BGN4 by constructing several expression vector systems using bifidobacterial promoters, a signal sequence, and a terminator. The pH and temperature stability and substrate specificity of the overexpressed β-glucosidase were characterized.

Materials and Methods

Bacterial Strains and Culture Conditions

The β-glucosidase-deficient *Escherichia coli* XL1 blue MR, *E. coli* DH5α, and *B. bifidum* BGN4 were used as cloning, subcloning, and expression hosts, respectively. *E. coli* XL1 blue MR and *E. coli* DH5α were cultured aerobically in Luria–Bertani (LB) broth (Becton Dickinson, Sparks, MD, USA) at 37°C for 15 h with vigorous shaking and supplemented with 100 μg/ml ampicillin (LBA; Sigma, St. Louis, MO, USA). If necessary. *Bifidobacterium* species were cultured anaerobically in brain heart infusion (BHI) broth (Becton Dickinson) supplemented with 0.05% (v/w) L-cysteine·HCl (Sigma), at 37°C for 16 h. The transformed *B. bifidum* BGN4 cells were grown in modified transgalactooligosaccharide propionate (TP) [17] broth supplemented with 1% (w/v) glucose instead of transgalactooligosaccharides (TOS), and 3.6 μg/ml chloramphenicol (BioBasic, Markham, ON, Canada) was added if necessary. The bacteria and plasmids used in this study are listed in Table 1.

Screening of Lactic Acid Bacteria for their β-Glucosidase Activities

Various lactic acid bacteria, including 21 *Bifidobacterium* spp., nine *Lactobacillus* spp., eight *Bacillus* spp., two *Lactococcus* spp., two *Enterococcus* spp., one *Leuconostoc* sp., two *Weissella* spp., and one *Pediococcus* sp., were used in this study (data not shown).

These bacteria were cultivated in BHI broth supplemented with 0.05% L-cysteine·HCl for 16 h at 37°C. An aliquot (1.0 ml) of culture broth was centrifuged at 6,000 ×*g* for 10 min at 4°C and washed twice with 500 μl of 50 mM phosphate buffer (PB, pH 6.0). The cells were resuspended in 500 μl of the same buffer and disrupted by sonication (VCX 400; Sonics & Materials Inc., Newton, CT, USA). The cell-free extracts were obtained by centrifugation at 6,000 ×*g* for 10 min at 4°C. The substrate specificity of each cell-free extract was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as the substrate (Sigma). An aliquot (90 μl) of each cell-free extract was mixed with 10 μl of 10 mM *p*NPG substrate and incubated at 37°C for the appropriate time. The reaction was stopped with 100 μl of 1 M Na_2CO_3 and the absorbance was measured at 405 nm. One unit of enzyme activity corresponded to the amount of enzyme that liberated 1 μmol of *p*-nitrophenol (*p*NP) per minute at 37°C. The amount of liberated *p*NP was calculated using a standard *p*NP solution (Sigma) and the protein concentration was measured with the Bradford method using bovine serum albumin (Bio-Rad, Piscataway, NJ, USA) as the standard, according to the manufacturer's instructions.

Table 1. Bacterial strains and plasmids.

Strain or vector	Relevant characteristics or genotype	Source or reference
Bacterial strains		
SH5	*B. lactis* SH5, wild type; Glu+ (original host of *bbg572*)	
RD68	*B. lactis* RD68, wild type; Glu+	
BGN4	*B. bifidum* BGN4, wild type; Glu-; Transformation host	
DH5α	*E. coli* DH5α, *F-(80dlacZ M15) (lacZYA-argF)U169hsdR17(r-m+) recA1 endA1 relA1 deoR*; Cloning host	[12]
XL1 blue MR	*E. coli* XL1 blue MR, *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*; Cloning host	Stratagene
Bpamyss572	*B. bifidum* BGN4 harboring pamyss572	
Bp919ss572	*B. bifidum* BGN4 harboring p919ss572	
Bp572ss572	*B. bifidum* BGN4 harboring p572ss572	
Bp919bbg572	*B. bifidum* BGN4 harboring p919bbg572	
Bp572bbg572	*B. bifidum* BGN4 harboring p572bbg572	
Bp572bbg572t	*B. bifidum* BGN4 harboring p572bbg572t	
Plasmids		
SuperCos1 Cosmid	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
pUC18	Amp^r	
pBES2	Amp^r, Cm^r; *E. coli–Bifidobacterium* shuttle vector[19]	[32]
pBESAF2	Amp^r, Cm^r; *E. coli–Bifidobacterium* shuttle vector[18]	[31]
pUC572PT	Amp^r: pUC18 with 2.276 kb insert of *B. lactis* DNA containing the *bbg572pt*	

Construction of Cosmid Library of *Bifidobacterium*

Chromosomal DNA was extracted from *Bifidobacterium* species with the method of Choi *et al*. [5] and partially digested with *Sau*3AI (Promega, Madison, WI, USA). The SuperCos 1 cosmid vector (Stratagene, La Jolla, CA, USA) was digested with *Bam*HI (Promega) and treated with calf intestinal alkaline phosphatase (Promega), and then ligated to the DNA fragments using a T4 DNA Ligation Kit (Stratagene). The ligation mixture was packaged *in vitro* using the Gigapack III Gold Packaging Extract (Stratagene) and transformed into *E. coli* XL1 blue MR according to the manufacturer's instructions. To select the bacterial clones expressing β-glucosidase activity, 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside (Wako Pure Chemical Industries, Osaka, Japan)-containing plates supplemented with ampicillin (Biobasic Inc., Toronto, ON, Canada) were used. Blue colonies were isolated as positive clones and used for further analysis. Plasmids were isolated from *E. coli* with the Midi Plus Ultrapure Plasmid Extraction System (Viogene, Taipei, Taiwan). The transformed *E. coli* cells were used to construct a genomic library.

Cloning of the *Bifidobacterium* β-Glucosidase Genes and Sequence Analysis

DNA fragments encoding β-glucosidase were isolated from a cosmid genomic library using Midi Plus (Viogene) and partially digested with *Bam*HI. These were subcloned into the cloning vector pUC18, digested with *Bam*HI, and used to transfect *E. coli* DHα as the cloning host, according to standard procedures [33].

The positive colonies showing β-glucosidase activity were isolated and the plasmids were purified and subjected to restriction and sequence analyses. The nucleotide sequences were determined with the BigDye Terminator v. 3.1 Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3730xl Genetic Analyzer (Applied Biosystems) in Bionicsro (Seoul, Korea). The promoter regions were predicted using the program at http://www.fruitfly.org/seq_tools/promoter.html. and the terminator regions were predicted with the program at http://linux1.softberry.com.

Analysis of Transcription Start Sites

The transcriptional start site of *bbg572* was determined by 5'-rapid amplification of cDNA ends (5'-RACE) using a RACE kit (Clontech, Palo Alto, CA, USA). mRNA from the *Bifidobacterium* species was reverse transcribed using SMARTScribe reverse transcriptase and 5'-CDS PrimerA provided by the manufacturer. After first-strand cDNA synthesis, the cDNA was tailed and amplified by PCR using a universal primer provided in the 5'-RACE kit and combined with the *bbg572*-gene-specific primer (5'-GGAATGACGCGCGGCACG CCAATCGAG-3'). PCR was performed under the following conditions: 5 min denaturation at 94°C, followed by 25 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 3 min; and the reaction was completed with a final extension at 72°C for 10 min. The PCR product was cloned into the pGEM-T Easy vector (Promega) and confirmed by sequencing.

Construction of a Vector Encoding β-Glucosidase and Transformation of *Bifidobacterium*

The target genes were amplified using *PfuUltra* II Fusion HS DNA polymerase (Stratagene) and each primer set (Table 2), according to the manufacturer's instruction manual.

First, the structural gene of the β-glucosidase, *bbg572*, was PCR amplified from the cosmid library using the appropriate primer set for each gene (Table 2) and cloned into the *Bifidobacterium* shuttle vector pBES2 [32] to construct pBES-bbg572. *B. bifidum* BGN4, a β-glucosidase-negative host, was transformed with this plasmid, according to the method of Kim *et al.* [21].

Second, to study the regulatory effects of the promoter and terminator sequence, regions of different gene promoters (*pamy*, *p919* and *p572*) and the region of the gene terminator (*572t*) were amplified from each template using the primer sets for each gene (Table 2). The amplified putative promoter sequences were digested with restriction enzymes and then cloned into pBES-bbg572 digested with the same restriction enzymes to incorporate the promoter upstream from the β-glucosidase gene. *B. bifidum* BGN4 was then transformed with the constructs individually. The amplified signal sequence (*ssamy*) was fused between the promoter and the structural gene in the corresponding vectors. The amplified putative terminator sequence of the β-glucosidase gene was digested with restriction enzymes and then cloned downstream from the structural gene in p572bbg572. *B. bifidum* BGN4 was then transformed with each construct individually. The 7 constructs are described in Table 3.

Table 2. Primers for amplifying the β-glucosidase gene and putative promoters from *Bifidobacterium*.

Primer name	Sequences of primer		Product	Reference
	Forward (5 → 3)	Reverse (5 → 3)		
primerbbg572pt	ggatccATGACGATGACGTTCCC	gaattcTGTGGGATGTTGTCCC	*bbg572pt*	
primerbbg572p	tctagaATGACGATGACGTTCCC	gaattcTGTGGGATGTTGTCCC	*bbg572p* (*p572+bbg572*)	
primerbbg572	ggatccATGACGATGACGTTCCC[a] agtactATGACGATGACGTTCCC[b]	gaattcTGTGGGATGTTGTCCC	*bbg572*	
primerbbg572t	ggatccATGACGATGACGTTCCC[a] agtactATGACGATGACGTTCCC[b]	gaattcTGTGGGATGTTGTCCC	*bbg572t* (*bbg572+572t*)	
primerPamy	tctagaGAAATACCGCAATGCACG	ggatccGGCTCCTTTATTCCTTTTC	*pamy*	[31]
primer919P	tctagaTGAAGTGTGTCGTGTGG	ggatccTGGTGTACCTTTTCTTG	*p919*	[37]
primer572P	tctagaATGCTGCTCCTTATGTGTC	ggatccTGCTGATTCCTCC	*p572*	
primerSSamy	ggatccATGAAACATCGGAAACC	agtact GGCCTGTGCTGCGG	*ssamy*	

Restriction enzyme sites are indicated by small letters: *Xba*I tctaga, *Bam*HI ggatcc, *Eco*RI gaattc, *Sca*I agtact. [a]Primers were used to construct p919bbg572, p572bbg572, and p572bbg572t. [b]Primers were used to construct pamyss572, p919ss572, p504ss572, and p572ss572.

Table 3. Constructed vectors with the cloned β-glucosidase gene.

Promoter			Signal sequences	ORF	Terminator	Plasmid
pamy	*p919*	*p572*	*ssamy*	*bbg572*	*572t*	
	o			o		p919bbg572
		o		o		p572bbg572
		o		o	o	p572bbg572t
o			o	o		pamyss572
	o		o	o		p919ss572
			o	o		p504ss572
		o	o	o		p572ss572

Analysis of β-Glucosidase Activity in Recombinant *B. bifidum* BGN4

The β-glucosidase activities of recombinant *B. bifidum* BGN4 were analyzed using *p*NPG as the substrate to compare the strengths of the promoters and terminator.

The recombinant *B. bifidum* BGN4 strains were cultured overnight at 37°C in modified TP broth supplemented with 3.6 μg/ml chloramphenicol. An aliquot (80 μl) of each culture broth was transferred to 8 ml of modified TP broth containing 3.6 μg/ml chloramphenicol and incubated for 16 h. The β-glucosidase activities were determined with *p*NPG, as described previously.

Optimal pH, Temperature, and Time of the β-Glucosidase Activity

The optimal pH for the recombinant β-glucosidase activity was determined with 10 mM *p*NPG as the substrate in 50 mM PB buffer at pH 3.0−8.0. *p*NPG (10 μl) was added to 90 μl of cell-free extract in each buffer [pH 2.0−8.0; 50 mM glycine-HCl (pH 2.0), 100 mM citric acid-citrate buffer (pH 3.0−6.0), 50 mM Tris-HCl buffer (pH 7.0−8.0)] and incubated at 37°C for 30 min. The reaction was stopped with 100 μl of 1 M Na_2CO_3 and the absorbance was measured at 405 nm.

To evaluate the optimal temperature for enzyme activity, the cell-free extract in 50 mM PB buffer (pH 6.0) was maintained at 10−80°C for 30 min, and the remaining activity was determined as described above.

The specific activity of β-glucosidase according to the culture timewas determined over a time range of 0−32 h. Each cell-free extract in 50 mM PB (pH 6.0) was maintained at 37°C for 30 min, and the activity was determined as described above.

Substrate Specificity of β-Glucosidase

Cellobiose, sophorose, laminaribiose, gentiobiose, daidzin, daidzein, genistin, glycitin, glycitein, and isoquercetrin were purchased from Sigma. Quercetin-3,4-di-O-β-D-glucoside and quercetin-7-O-β-D-glucoside were purchased from Extrasynthese (Genay, France). Genistein and quercetin-7-glucoside were purchased from Chengdu Biopurify Phytochemicals Ltd (Chengdu, China). Ginsenoside standards such as compound K, Rb1, Rb2, Rc, Rd, F1, Rg3, and Rh1 were purchased from Cogon Chemical (Chengdu, China); F2, Rg2(S), and Rh2(s) were from LKT Laboratories (St. Paul, MN, USA); Rg1 was from Wako Pure Chemical Industries; Rg3 (S) and Re were from BTGin Co. Ltd (Daejeon, Korea). All standard ginsenosides were dissolved in water (containing 3% Tween 80) as individual solutions, and saccharides were dissolved in water. Other substrates were dissolved in methanol (J.T. Baker, Phillipsburg, NJ, USA).

The cell-free extract from the overnight culture of the recombinant *B. bifidum* BGN4 was prepared in 50 mM PB (pH 6.0), as described above. Reaction mixtures (200 μl) containing 180 μl of cell-free extract and 1 mg/ml ginsenosides were incubated at 37°C with shaking. Samples were harvested at 24 h and analyzed with thin-layer chromatography (TLC), according to Chi and Ji [4].

For the hydrolysis of disaccharides, 180 μl of cell-free extract in 50 mM PB (pH 6.0) was mixed with 20 μl of 100 mM disaccharide in the same buffer and incubated at 37°C. At 24 h, the samples were analyzed with TLC using *n*-butanol:formic acid:water (4:8:1) as the developer. They were visualized by spraying with 10% H_2SO_4 and drying at 120°C for 10 min.

For the hydrolysis of the flavonoid glycosides, 180 μl of cell-free extract in 50 mM PB (pH 6.0) was mixed with 20 μl of 100 mM quercetin and incubated at 37°C. After 24 h, the samples were analyzed with TLC using *n*-butanol:acetic acid:water (7:1:2) as the developer. The samples were visualized by spraying them with 10% H_2SO_4 and drying at 120°C for 10 min.

For the hydrolysis of the isoflavone glycosides, 180 μl of cell-free extract in 50 mM PB (pH 6.0) was mixed with 20 μl of 100 mM isoflavone and incubated at 37°C. After 24 h, the samples were analyzed with TLC using chloroform:methanol:water (65:35:10) as the developer. The isoflavones were visualized under a UV lamp at 254 nm.

Results and Discussion

Sequence Analysis of *Bifidobacterium* β-Glucosidase Genes

Two *B. lactis* strains (SH5 and RD68) showing relatively high β-glucosidase activities were selected from 46 experimental lactic acid bacterial strains. After the construction of cosmid libraries in *E. coli* using the genomic DNAs from these two strains, about 30 clones with β-glucosidase activity were isolated for each construct. One clone from each screened colony showing the greatest enzyme activity was selected. The fragment insert in the cosmid was digested with *Bam*HI and subcloned into pUC18, and the β-glucosidase-positive clones were isolated. Sequence analysis of the two cloned β-glucosidase genes

showed that the β-glucosidase gene from *B. lactis* SH5 was identical to that of *B. lactis* RD68 with respect to its open reading frame (ORF), putative promoter region, and putative terminator region. As a consequence, the β-glucosidase gene from *B. lactis* SH5 was designated as *bbg572pt* and used for further study (Fig. 1). *bbg572pt* comprised 2,276 bp and contained one ORF. A putative terminator sequence was located downstream of the ORF. The putative ribosome binding site (RBS), AGGAGGA, was detected (gray box; Fig. 1) upstream of the ORF, which is similar to the RBS of *B. longum* MG1 [30]. The deduced amino acid sequence contained 460 residues, with a molecular mass of 50.71 kDa, with 85%, 68%, 70%, and 69% amino acid sequence identities with the β-glucosidases from *B. breve* clb, *B. breve* CECT7263, *B. dentium* ATCC27679, and *B. longum* subsp. *infantis* ATC15697, respectively (Fig. 2). Based on these amino acid sequence similarities, the β-glucosidase encoded by

```
1     atgctgctcc  ttatgtgtct  ctgctgtgcc  gattatatgc  ctattctcgc  ggtgtcgcgg  ccctcacttt  gctgcgaaac
81    tctgttgcga  actctgttac  gggccaagtt  tgtgcgtacg  ggctaggatt  ttcggttacg  ggctagggca  tctctgcctt
161   ttcgtgaaaa  acgggtagtc  ggaaaccgcc  atgttccaat  gatcccgttt  gccggggccc  cgactttctt  tagcctgtaa
241   cgcaaaatcg  tagcccgtac  gcacaaactt  ggcccgtaag  caagaagtta  gcccgtaaga  gatggggaat  ctggcccgta
321   acggatggaa  gagagcgggg  gggatgtgga  agattgttgg  cgatgcgcgt  gttgcgggcg  gtgtggaggg  gaggagcatg
401   ctatatttgg  catAtgctga  ctcgagagta  agtaaatgaa  tgccggagca  gatccgggat  ttgcgcgggt  ctgtgctcgg
481   atgcgacgtg  tattgtGtcg  gatgtatcga  cAGGAGGAat  cagca
526   atgacgatgacgttcccgaagggcttccagttcggcaccgcgactgccgcctaccagatcgaaggcgcggtggacgaagacggccgcacgccg
        M  T  M  T  F  P  K  G  F  Q  F  G  T  A  T  A  A  Y  Q  I  E  G  A  V  D  E  D  G  R  T  P
619   tcgatctgggatgtgttctcgcacgccccgggccgcgtgctgaatggcgacaccggagacaaggccgacgatttctaccaccgctggcaggac
        S  I  W  D  V  F  S  H  A  P  G  R  V  L  N  G  D  T  G  D  K  A  D  D  F  Y  H  R  W  Q  D
712   gatctcaagctcgtgcgcgatctcggcgtgaacgcataccggttctcgattggcgtgccgcgcgtcattcccaccccggacggcaagccgaac
        D  L  K  L  V  R  D  L  G  V  N  A  Y  R  F  S  I  G  V  P  R  V  I  P  T  P  D  G  K  P  N
805   gagaagggcctcgatttctacgagcgcattgtcgaccagctgctcgaatacggcatcgacccgattgtgacgctctaccattgggatctgccg
        E  K  G  L  D  F  Y  E  R  I  V  D  Q  L  L  E  Y  G  I  D  P  I  V  T  L  Y  H  W  D  L  P
898   cagtatctgaacgaagatccgtaccgggatggctggctgaaccgtgagaccgcgttccgcatggcggagtatgccggcattgtggccaagcgc
        Q  Y  L  N  E  D  P  Y  R  D  G  W  L  N  R  E  T  A  F  R  M  A  E  Y  A  G  I  V  A  K  R
991   ctcggcgaccgtgtgcacacctacacacgctcaacgaaccgtggtgctcggcgcacctgagctacggcggcaccgagcatgcccccggcctg
        L  G  D  R  V  H  T  Y  T  T  L  N  E  P  W  C  S  A  H  L  S  Y  G  G  T  E  H  A  P  G  L
1084  ggcgccggcccgctcgcgttccgcgccgcccatcacctgaatctggcacatggtctgatgtgcgaggcagtgcgtgccgaggccggagcgaag
        G  A  G  P  L  A  F  R  A  A  H  H  L  N  L  A  H  G  L  M  C  E  A  V  R  A  E  A  G  A  K
1177  ccggatctctcggtgacgctgaatctgcaggtgaaccgcggtgatgcggatgccgtgcaccgcgtggatctcattgccaaccgcgtgttcctc
        P  D  L  S  V  T  L  N  L  Q  V  N  R  G  D  A  D  A  V  H  R  V  D  L  I  A  N  R  V  F  L
1270  gatccgatgctgcgcggctactacccggacgagctgttcgcaatcaccaagggaatctgcgattgggacttcgtgcatgacggcgatctcaag
        D  P  M  L  R  G  Y  Y  P  D  E  L  F  A  I  T  K  G  I  C  D  W  D  F  V  H  D  G  D  L  K
1363  ctcatcaaccagccgattgacgtcctggggcttaattattactcgacgaatctgctcgccatgagcgaccgcccgcagttcccgcagagcacc
        L  I  N  Q  P  I  D  V  L  G  L  N  Y  Y  S  T  N  L  L  A  M  S  D  R  P  Q  F  P  Q  S  T
1456  gaggcctccaccgcgccgggcgccagcgacatcgactggctgcctaccgacggcccgcacacgcagatggggtggaacatcgacccggatgcg
        E  A  S  T  A  P  G  A  S  D  I  D  W  L  P  T  D  G  P  H  T  Q  M  G  W  N  I  D  P  D  A
1549  ctttataacacgctggttcgcctgaacgacgactacgaccacattccgctcgtcgtcactgaaaacggcatggcgtgccccgacgaggtggaa
        L  Y  N  T  L  V  R  L  N  D  D  Y  D  H  I  P  L  V  V  T  E  N  G  M  A  C  P  D  E  V  E
1642  gtcggcccggatggtgtgaagatggtgcacgacgacgaccgcatcgactacctgcgtcgccatctcgaggccgtccaccgcgcgatcgaggag
        V  G  P  D  G  V  K  M  V  H  D  D  D  R  I  D  Y  L  R  R  H  L  E  A  V  H  R  A  I  E  E
1735  ggggcgaatgtcatcggatacttcgtgtggtcgctgatggataatttcgagtgggcgttcggctacgaccgccgcttcggcctgacctacgtg
        G  A  N  V  I  G  Y  F  V  W  S  L  M  D  N  F  E  W  A  F  G  Y  D  R  R  F  G  L  T  Y  V
1828  gactacgacaccgaggagcgcatacggaaggacagctacaactggtaccgtaacttcatcgccgagcactccgccaagtag
        D  Y  D  T  E  E  R  I  R  K  D  S  Y  N  W  Y  R  N  F  I  A  E  H  S  A  K  *
1909  cgggttcggg  cgcgccgggc  gcgggtcatg  caatgtctgt  ggatatgcac  atgtatgagc  atgcacagac  gagttgcata
1989  ttcgcgcccg  gctcgttttg  atttcaggat  gtggacggcg  cgtcgcctga  agcgattaac  tatttgggat  tacttgcaga
2069  gaatctcacc  cagttttgtg  tgacggtcta  ataaatttga  aatccgcgga  aattcgggaa  agcgcggcgg  agaggtaatt
2149  tgcggcggtg  tgaaatctga  ggcaaattgg  ctcggtaaaa  gaacttaagc  gcttgataaa  ctcgggtgag  gggaattatt
2229  ctttcaatca  tcgattcgca  agcccgcgaa  gctgcgggga  gcgaattc
```

Fig. 1. Nucleotide and deduced amino acid sequences of the *bbg572pt* in pUC18 (pUC572PT).
The putative terminator sequence is indicated by bold letters. The putative ribosomal binding site (RBS) is indicated in the gray box and capitalized. Transcription start points are in bold and capitalized. The predicted promoter region is underlined. The translational termination site is marked by an asterisk. The sequence data are available in GenBank under the accession number JX274651.

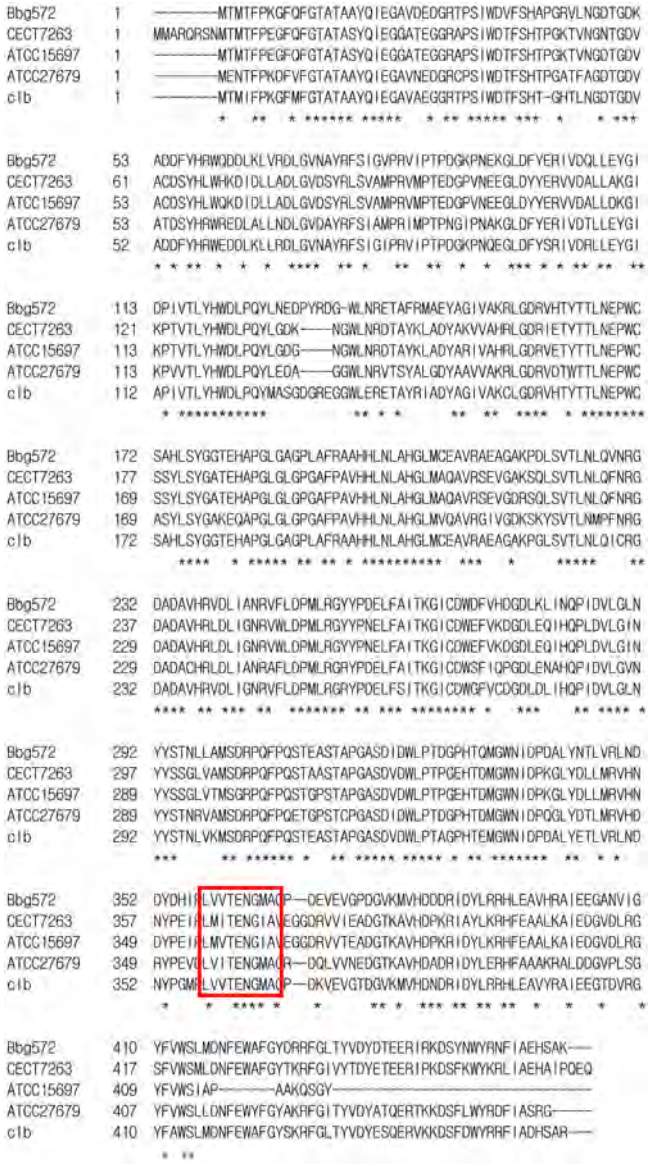


Fig. 2. Multiple sequence alignment of β-glucosidases from *Bifidobacterium*.
Bbg572, *Bifidobacterium lactis* SH5 and RD68; CECT7263, *Bifidobacterium breve* CECT 7263; ATCC15697, *Bifidobacterium longum* subsp. *infantis* ATCC 15697; ATCC27679, *Bifidobacterium dentium* ATCC 27679; clb, *Bifidobacterium breve* clb. A region conserved in glycosyl hydrolase family 3 is boxed. Conserved amino acid residues are indicated by asterisks.

bbg572 was assigned to glycosyl hydrolase family 1. The overall GC content of *Bifidobacterium* is generally 55.0%–67.0%, which is higher than those of other bacteria [35]. The GC content of *bbg572* was 63%, which is slightly lower than that of the β-glucosidase from *B. breve* clb (65.1%) [28].

Analysis of the Transcription Start Point

The putative promoter was predicted upstream from *bbg572* (Fig. 1) and designated as *p572*. The putative −10 and −35 positions in the promoter region showed homology with the consensus sequence of *E. coli*, and this promoter induced successful expression of *bbg572* in *E. coli* DH5α (data not shown). 5'-RACE analysis revealed two potential transcription start points (*tsp*s) for *bbg572* (the G and A residues in Fig. 3). The −10 region (tgtatt) and −35 region (ttgcgc) upstream from the G residue displayed 66.7% and 50% identity, respectively, to the corresponding *E. coli* consensus sequences. The −10 region (tatatt*)* and −35 region (ttgcgg) upstream from the A residue displayed 83.3% and 50% sequence identity, respectively, to the corresponding *E. coli* consensus sequences.

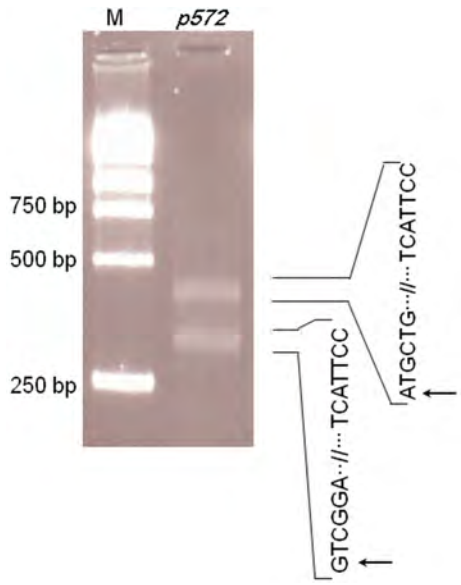


Fig. 3. 5'-Rapid amplification of cDNA ends (RACE) result of the *bbg572*.
Lane M, 1 kb DNA ladder. A labeled 27-mer oligonucleotide primer complementary to nucleotide 756–782 (257 bp from the initiation codon) of the *bbg572pt* was utilized to define the transcription start points (*tsp*s) indicated in lane 2. The *tsp*s are indicated by arrows.

The divergence in sequence homology in the −35 regions is consistent with many previous studies that have shown that transcription readily occurs in *E. coli* even in the absence of absolute similarity in this region [6].

Expression of β-Glucosidase in *B. bifidum* BGN4

Strong promoters are required for the enhanced expression of a target gene. In our previous studies, we have successfully expressed foreign genes in *B. longum* using several promoters, including *p919* from *B. bifidum* BGN4 [37] and *pamy* from the α-amylase gene of *B. pseudocatenulatum* INT57 [31]. To compare the strengths of these promoters, several vectors were constructed with combinations of promoters and reporter (*bbg572*) and transformed into the β-glucosidase-negative strain *B. bifidum* BGN4 as the host. Promoter strength was analyzed by measuring the β-glucosidase activity using *p*NPG.

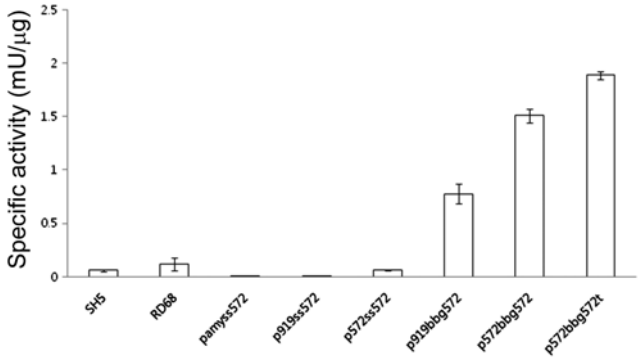


Fig. 4. Specific β-glucosidase activities of wild-type *Bifidobacterium* spp. and recombinant *B. bifidum* BGN4 with various vector constructs.
The total β-glucosidase activity was determined at 37°C in the 50 mM Tris-HCl, pH 6.0, with ρNPG as a substrate. SH5, *B. lactis* SH5; RD68, *B. lactis* RD68; others, *B. bifidum* BGN4 harboring each vector.

When the signal sequence of the amylase gene (*ssamy*) was inserted between the promoter and reporter gene to induce the extracellular expression of *bbg572*, all of the transformants containing *ssamy* showed unexpectedly low β-glucosidase activities in *B. bifidum* BGN4 (Fig. 4). The *ssamy* also failed to export the *bbg572* products expressed in *B. bifidum* BGN4. The intracellular β-glucosidase activity of Bp572ss572 was 1,186-fold higher than its extracellular activity (data not shown). This might be attributable to the incompatibility between the transformation host and the promoters and *ssamy* used. We assumed that the change in transformation host affected the activities of the promoters and *ssamy*, causing low protein expression and secretion. Many previous studies have shown that the −10 and −35 sequences affect the promoter strength [26]. Other factors, such as the TG motif [14], spacers, UP elements [9], and the promoter's three-dimensional structure, are also reported to affect the efficiency of transcription [16].

In the expression system for *bbg572*, *p572* was a stronger promoter than *pamy* or *p919* (Fig. 4). In the expression systems for *bbg572*, the expression of β-glucosidase was markedly enhanced by the deletion of the *ssamy*. The removal of *ssamy* resulted in a 24.35-fold increase in Bp572bbg572 (1.5 mU/μg protein) and a 137-fold increase in that of Bp919bbg572 (0.78 mU/μg protein) (Fig. 4).

It is well known that the transcription terminator enhances the expression of a gene [1]. A putative transcription terminator was found downstream of *bbg572* and designated as *572t*. It was added downstream of the *bbg572* in p572bbg572 to produce p572bbg572t. The addition of the terminator caused a 25.4% increase in the specific β-glucosidase activity in *B. bifidum* BGN4 (Fig. 4). The β-glucosidase activity of *B. bifidum* BGN4 harboring p572*bbg572t* was 1.89 mU/μg, which was much higher than those of *B. lactis* SH5 (31.32-fold) and *B. lactis* RD68 (15.88-fold) (Fig. 4).

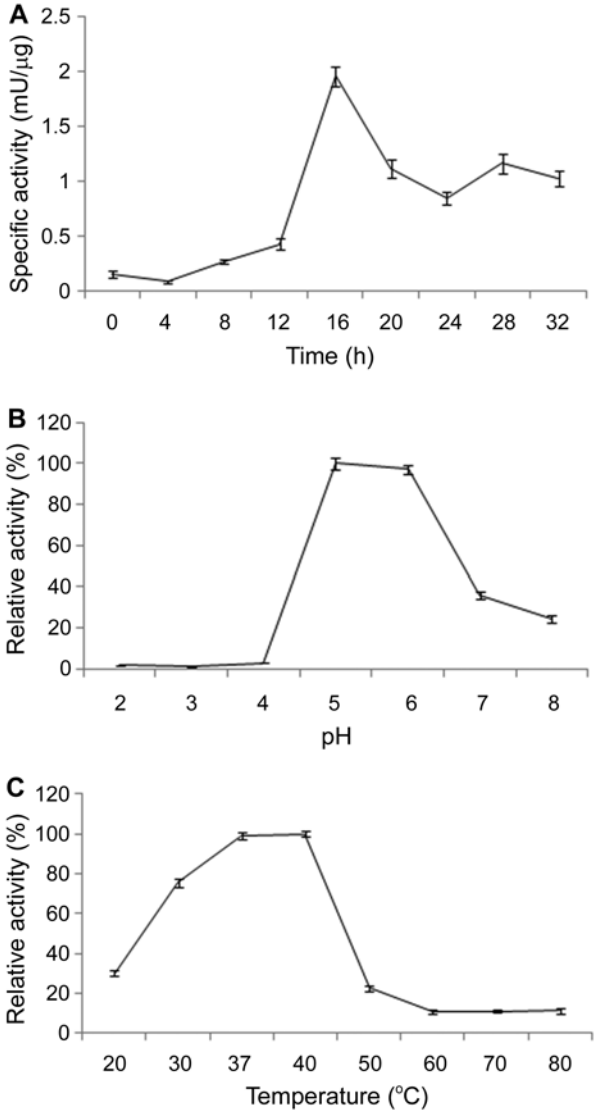


Fig. 5. Specific β-glucosidase activities of Bp572bbg572t (**A**) according to the culture time, and relative β-glucosidase activities of Bp572bbg572t at pH range from 2 to 8 (**B**) and temperature range from 20°C to 80°C (**C**).
The cell-free extract of Bp572bbg572t was used as enzyme and ρNPG as a substrate.

Biochemical Characterizations of the β-Glucosidase Activity of Cell Extracts from Transformants Harboring p572bbg572t

The β-glucosidase activity of Bp572bbg572t has been analyzed according to culture time, pH, and temperature. As shown in Fig. 5A, the specific activity increased according to the culture time, peaking at 16 h and decreasing slowly thereafter.

The optimal pH for Bp572bbg572t was pH 6.0 (Fig. 5B), which is consistent with those of most bacterial β-glucosidases, which have pH optima in slightly acidic or neutral pH ranges [10]. In previous studies, the optimal pHs of the β-glucosidases from *B. breve* 203, β-D-glucosidase I and β-D-glucosidase II, were 6.0 and 5.5, respectively [20], and that of the β-glucosidase of *B. breve* clb was pH 5.5 [28].

When the enzyme activity was investigated in the temperature range of 20–80°C, Bp572bbg572t exhibited more than 95% of its maximal activity at 30–50°C, with a sharp decrease above 50°C (Fig. 5C).

In the previous study, the optimal temperatures of the β-glucosidase enzymes from *B. breve* 203, β-D-glucosidase I and β-D-glucosidase II, were 45°C and 40°C, respectively [20], and that of the β-glucosidase from *B. breve* clb was 45°C [28].

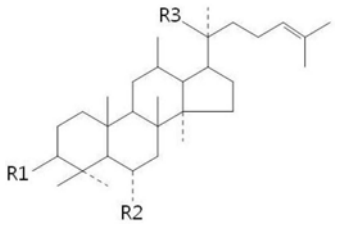
Substrate Specificity of the Recombinant β-Glucosidase from *B. bifidum* BGN4

The substrate range of Bp572bbg572t was investigated using various disaccharides, isoflavones, quercetins, and ginsenosides (Tables 4 and 5). As a control, the cell extract of *B. bifidum* BGN4 harboring pBESAF2 [31] showed no detectable glycosidic activity on various substrates (data not shown).

Table 4. Substrate specificities of Bp572bbg572t.

Substrate	Linkage	Enzyme activity
		Bbg572
Disaccharides		
Cellobiose	*O*-β-D-glucosyl-(1→4)-D-glucose	O
Sophorose	*O*-β-D-glucosyl-(1→2)-D-glucose	O
Laminaribiose	*O*-β-D-glucosyl-(1→3)-D-glucose	O
Gentiobiose	*O*-β-D-glucosyl-(1→6)-D-glucose	O
Isoflavone		
Daidzin	Daidzein 7-*O*-β-D-glucoside	O
Genistin	Genistein 7-*O*-β-D-glucoside	O
Glycitin	Glycitein 7-*O*-β-D-glucoside	O
Quercetin		
Isoquercetrin	Quercetin-3-*O*-β-D-glucoside	O
Spiraeoside	Quercetin-4-*O*-β-D-glucoside	O
	Quercetin-3,4-di-*O*-β-D-glucoside	O
	Quercetin-7-*O*-β-D-glucoside	O

Table 5. Substrate specificity of Bp572bbg572t on various ginsenosides.

Ginsenoside	R1	R2	R3	Enzyme activity
				Bbg572
				
20(*S*)-Protopanaxadiol type				
Compound K	OH	H	O-Glc[a]	×
F2	O-Glc	H	O-Glc	×
Rb1	O-Glc^{2}-^{1}Glc[a]	H	O-Glc^{6}-^{1}Glc	O
Rb2	O-Glc^{2}-^{1}Glc[a]	H	O-Glc^{6}-^{1}Arap[a]	O
Rc	O-Glc^{2}-^{1}Glc[a]	H	O-Glc^{6}-^{1}Araf[a]	×
Rd	O-Glc^{2}-^{1}Glc[a]	H	O-Glc[a]	×
20(*S*)-Protopanaxatriol type				
F1	OH	OH	O-Glc[a]	×
Re	OH	O-Glc^{2}-^{1}Rha[a]	O-Glc[a]	×
Rg1	OH	O-Glc[a]	O-Glc[a]	×
Rg2(*S*)	OH	O-Glc^{2}-^{1}Rha[a]	OH	×
Rg3(*S*)	O-Glc-Glc[a]	H	H	×
Rg3(*R*)	O-Glc-Glc[a]	H	H	×
Rh1	OH	O-Glc[a]	OH	×
Rh2(*S*)	O-Glc[a]	H	H	×

[a]Arap, α-L-arabinopyranosyl; Araf, α-L-arabinofuranosyl; Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl.

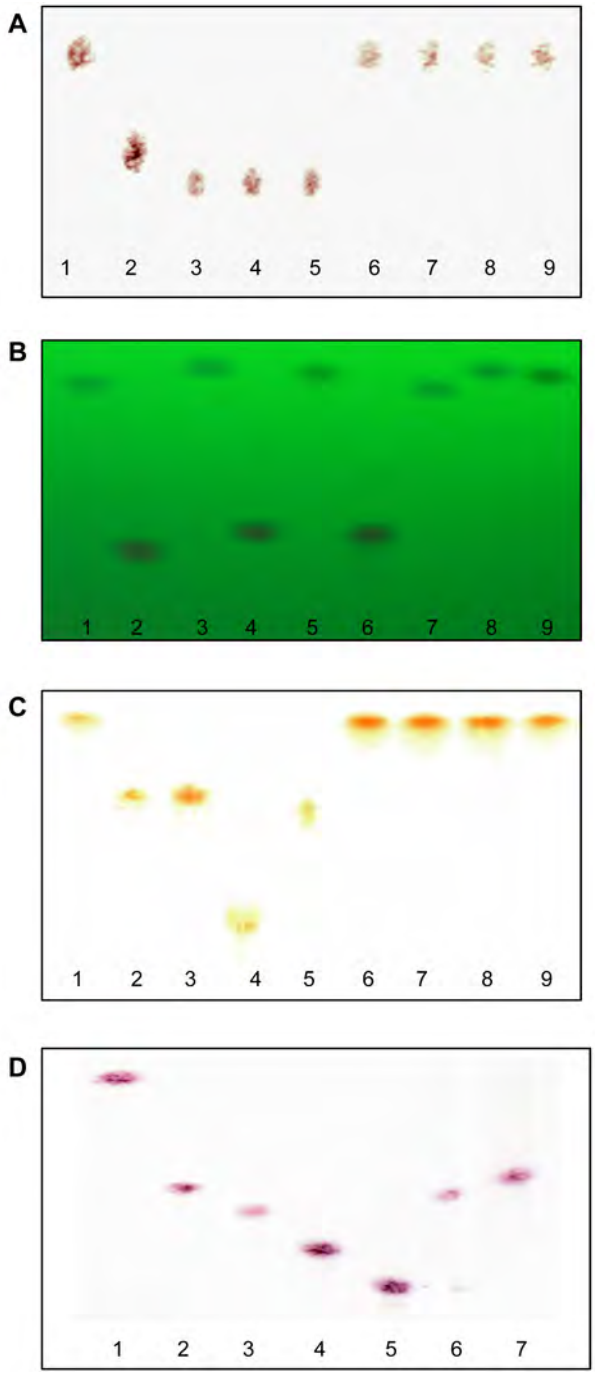


Fig. 6. TLC chromatogram of the hydrolysis product of various disaccharides (**A**), isoflavones (**B**), quercetins (**C**), and ginsenosides using cell freee extract of Bp572bbg572t.
(**A**) 1, Glucose; 2, cellobiose; 3, sophorose; 4, laminaribiose; 5, gentiobiose; Lanes 6 to 9, hydrolysis products of cellobiose (lane 6), sophorose (lane 7), laminaribiose (lane 8), and gentiobiose (lane 9). (**B**) 1, Daidzein; 2, daidzin; 3, glycitein; 4, glycitin; 5, genistein; 6, genistin; Lanes 7 to 9, hydrolysis products of daidzin (lanes 7), glycitin (lanes 8), and genistin (lanes 9). (**C**) 1, Quercetin; 2, isoquercetrin; 3, spiraeoside; 4, isoquercitrin; 5, quercetin-7-*O*-β-D-glucoside; Lanes 6 to 9, hydrolysis products of isoquercetrin (lanes 6), spiraeoside (lane 7), isoquercitrin (lane 8) and quercetin-7-O-β-D-glucoside (lane 9). (**D**) 1, Rg3; 2, Rd; 3, Re; 4, Rb2; 5, Rb1; Lanes 6 to 7, hydrolysis products of Rb1 (lanes 6), and Rb2 (lane 7).

Bp572bbg572t was able to hydrolyze β-1,2 (sophorose), β-1,3 (laminaribiose), β-1,4 (cellobiose), and β-1,6 (gentiobiose) linkages between two glucose molecules. It showed β-glucosidase activities on the ginsenoside Rb1, Rb2, and seven flavonoids (daidzin, genistin, glycitin, isoquercetrin, spiraeoside, quercetin-3,4-di-O-β-D-glucoside, and quercetin-7-O-β-D-glucoside). No release of hydrolysis products by Bp572bbg572t was observed for the protopanaxadiol-type ginsenosides compound K, Rc, and Rd, or the protopanaxatriol-type ginsenosides F1, Re, Rg1, Rg2(*S*), Rg3(*S*), Rg3(*R*), Rh1, and Rh2(*S*) (data not shown).

β-Glucosidases are used to hydrolyze phenolic compounds and phytoestrogen glucosides to improve their biological activity, and have several applications in the field of medicine, and in the food industry [2]. In this study, we focused on the various glycoside specificities of recombinant β-glucosidase (Bp572bbg572t) and its full potential exploitation to increase the activity of β-glucosidase. The *bbg572* encodes an enzyme of glycosyl hydrolase family 1 and selectively catalyzes the cleavage of glucosidic bonds.

In this study, we found that the highest expression level of *bbg572* in *B. bifidum* BGN4 was achieved when using its own promoter and terminator. Furthermore, the expression level was 31.32-fold higher than that of the original strain *B. lactis* SH5. Furthermore, our results of bioconversion of several glycosidic phytochemicals suggest that these new β-glucosidase-positive transformants of *B. bifidum* BGN4 can be utilized for the production of specific aglycone products for the food industry and many biotechnological applications.

Acknowledgments

This work was supported by the Next-Generation BioGreen 21 Program (No.PJ008005), Rural Development Administration, and by the Small and Medium Business Administration (SA114187), Republic of Korea.

References

1. Aiba, H., A. Hanamura, and H. Yamano. 1991. Transcriptional terminator is a positive regulatory element in the expression of the *Escherichia coli crp* gene. *J. Biol. Chem*. **266:** 1721–1727.
2. Bhatia, Y., S. Mishra, and V. S. Bisaria. 2002. Microbial β-glucosidases: Cloning, properties and applications. *Crit. Rev. Biotechnol*. **22:** 375–407.
3. Brown, J. P. 1998. Hydrolysis of glycosides and esters. *In: Role of the Gut Flora in Toxicity and Cancer*. Academic Press, San Diego.
4. Chi, H. and G. E. Ji. 2005. Transformation of ginsenosides Rb1 and Re from *Panax ginseng* by food microorganisms. *Biotechnol. Lett*. **27:** 765–771.

5. Choi, Y. O., J. M. Seo, and G. E. Ji. 2008. Modulatory activity of CpG oligonucleotides from *Bifidobacterium longum* on immune cells. *Food Sci. Biotechnol.* **17:** 1131−1395.
6. Collado-Vides, J., B. Magasanik, and J. D. Gralla. 1991. Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol. Rev.* **55:** 371−394.
7. Mahlen, S. D. and J. E. Clarridge. 2009. Site and clinical significance of *Alloscardovia omnicolens* and *Bifidobacterium* species isolated in the clinical laboratory. *J. Clin. Microbiol.* **47:** 3289−3293.
8. Esen, A. 1993. β-Glucosidase, pp. 1−13. *In: β-Glucosidase: Biochemistry and Molecular Biology.* American Chemical Society, Washington DC.
9. Estrem, S. T., T. Gaal, W. Ross, and R. L. Gourse. 1998. Identification of an UP element consensus sequence for bacterial promoters. *Proc. Natl. Acad. Sci. USA* **95:** 9761−9766.
10. Gekas, V. and M. H. Lopez-Levia. 1985. Hydrolysis of lactose. *Process Biochem.* **20:** 2−12.
11. Ghosh, P., N. B. Pamment, and W. R. B. Martin. 1982. Simultaneous saccharification and fermentation of cellulose: Effect of beta-D-glucosidase activity and ethanol inhibition of cellulases. *Enzyme Microb. Technol.* **4:** 425−430.
12. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557−580.
13. Hawkswor, G., B. S. Drasar, and M. J. Hill. 1971. Intestinal bacteria and hydrolysis of glycosidic bonds. *J. Med. Microbiol.* **4:** 451−459.
14. Helmann, J. D. 1995. Compilation and analysis of *Bacillus subtilis* sigma A-dependent promoter sequences: Evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res.* **23:** 2351−2360.
15. Hendrich, S. 2002. Bioavailability of isoflavones. *J. Chromatogr. B* **777:** 203−210.
16. Jensen, P. R. and K. Hammer. 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl. Environ. Microbiol.* **64:** 82−87.
17. Ji, G. E., S. K. Lee, and I. H. Kim. 1994. Improved selective medium for isolation and enumeration of *Bifidobacterium* sp. *Korean J. Food Sci. Technol.* **26:** 526−531.
18. Joint FAO/WHO Expert Consultation. 2001. Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.
19. Kawakami, Y., W. Tsurugasaki, S. Nakamura, and K. Osada. 2005. Comparison of regulative functions between dietary soy isoflavones aglycone and glucoside on lipid metabolism in rats fed cholesterol. *J. Nutr. Biochem.* **16:** 205−212.
20. Kenji, S., T. Takashi, K. Hidehiko, and T. Tatsurokuro. 1986. Isolation and characterization of two β-D-glucosidases from *Bifidobacterium breve* 203. *Agric. Biol. Chem.* **50:** 2287−2293.
21. Kim, J. Y., Y. Wang, M. S. Park, and G. E. Ji. 2010. Improvement of transformation efficiency through *in vitro* methylation and *Sac*II site mutation of plasmid vector in *Bifidobacterium longum* MG1. *J. Microbiol. Biotechnol.* **20:** 1022−1026.
22. Kim, J. Y., Y. Wang, S. J. Park, M. S. Park, and G. E. Ji. 2012. Cloning of expression of β-glucosidases from *Bifidobacterium lactis* AD011. *Food Sci. Biotechnol.* **21:** 731−738.
23. Le, T. M. and N. T. Vu. 2010. Cloning of a β-glucosidase gene (BGL1) from traditional starter yeast *Saccharomycopsis fibuligera* BMQ 908 and expression in *Pichia pastoris*. *Int. J. Biol. Life Sci.* **6:** 83−87.
24. Lei, V., W. K. Amoa-Awua, and L. Brimer. 1999. Degradation of cyanogenic glycosides by *Lactobacillus plantarum* strains from spontaneous cassava fermentation and other microorganisms. *Int. J. Food Microbiol.* **53:** 169−184.
25. Marotti, I., A. Bonetti, B. Biavati, P. Catizone, and G. Dinelli. 2007. Biotransformation of common bean (*Phaseolus vulgaris* L.) flavonoid glycosides by *Bifidobacterium* species from human intestinal origin. *J. Agric. Food Chem.* **55:** 3913−3919.
26. McCracken, A., M. S. Turner, P. Giffard, L. M. Hafner, and P. Timms. 2000. Analysis of promoter sequences from *Lactobacillus* and *Lactococcus* and their activity in several *Lactobacillus* species. *Arch. Microbiol.* **173:** 383−389.
27. Nunoura, N., K. Ohdan, K. Tanaka, H. Tamaki, T. Yano, M. Inui, *et al*. 1996. Cloning and nucleotide sequence of the beta-D-glucosidase gene from *Bifidobacterium breve* clb, and expression of beta-D-glucosidase activity in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **60:** 2011−2018.
28. Nunoura, N., K. Ohdan, T. Yano, K. Yamamoto, and H. Kumagai. 1996. Purification and characterization of beta-D-glucosidase (beta-D-fucosidase) from *Bifidobacterium breve* clb acclimated to cellobiose. *Biosci. Biotechnol. Biochem.* **60:** 188−193.
29. Nunoura, N., K. Ohdan, K. Yamamoto, and H. Kumagai. 1997. Expression of the β-d-glucosidase I gene in *Bifidobacterium breve* 203 during acclimation to cellobiose. *J. Ferment. Bioeng.* **83:** 309−314.
30. Park, M. S., B. Kwon, J. J. Shim. C. S. Huh, and G. E. Ji. 2008. Heterologous expression of cholesterol oxidase in *Bifidobacterium longum* under the control of 16S rRNA gene promoter of bifidobacteria. *Biotechnol. Lett.* **30:** 165−172.
31. Park, M. S., J. M. Seo, and J. Y. Kim. 2005. Heterologous gene expression and secretion in *Bifidobacterium longum*. *Lait* **85:** 1−8.
32. Park, M. S., H. W. Moon, and G. E. Ji. 2003. Molecular characterization of plasmid from *Bifidobacterium longum*. *J. Microbiol. Biotechnol.* **13:** 457−462.
33. Sambrook, J., E. F. Frietsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
34. Setchell, K. D. R., N. M. Brown, L. Zimmer-Nechemias, W. T. Brashear, B. E. Wolfe, A. S. Kirschner, and J. E. Heubi. 2002. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am. J. Clin. Nutr.* **76:** 447−453.
35. Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt. 1986. *Bergey's Manual of Systematic Bacteriology.* The Williams & Wilkins Co.
36. Ventura, M., F. Turroni, A. Zomer, E. Foroni, V. Giubellini, F. Bottacini, *et al*. 2009. The *Bifidobacterium dentium* Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. *PLoS Genet.* **5:** e1000785.
37. Wang, Y., J. Y. Kim, M. S. Park, and G. E. Ji. 2012. Novel *Bifidobacterium* promoters selected through microarray analysis lead to constitutive high level expression. *J. Microbiol.* **50:** 638−643.
38. Xu, X., K. S. Harris, H. J. Wang, P. A. Murphy, and S. Hendrich. 1995. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.* **125:** 2307−2315.

THE GERONTOLOGICAL SOCIETY OF AMERICA®

Journals of Gerontology: Biological Sciences
cite as: *J Gerontol A Biol Sci Med Sci*, 2020, Vol. XX, No. XX, 1–9
doi:10.1093/gerona/glaa090
Advance Access publication April 17, 2020

OXFORD

Original Article

Probiotic Supplementation Improves Cognitive Function and Mood with Changes in Gut Microbiota in Community-Dwelling Older Adults: A Randomized, Double-Blind, Placebo-Controlled, Multicenter Trial

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Received: January 19, 2020; Editorial Decision Date: April 6, 2020

Decision Editor: David Le Couteur, MBBS, FRACP, PhD

Abstract

Probiotics have been proposed to ameliorate cognitive impairment and depressive disorder via the gut–brain axis in patients and experimental animal models. However, the beneficial role of probiotics in brain functions of healthy older adults remains unclear. Therefore, a randomized, double-blind, and placebo-controlled multicenter trial was conducted to determine the effects of probiotics on cognition and mood in community-dwelling older adults. Sixty-three healthy elders (≥65 years) consumed either placebo or probiotics containing *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI for 12 weeks. The gut microbiota was analyzed using 16S rRNA sequencing and bioinformatics. Brain functions were measured using the Consortium to Establish a Registry for Alzheimer's disease, Satisfaction with life scale, stress questionnaire, Geriatric depression scale, and Positive affect and negative affect schedule. Blood brain-derived neurotrophic factor (BDNF) was determined using enzyme-linked immunosorbent assay. Relative abundance of inflammation-causing gut bacteria was significantly reduced at Week 12 in the probiotics group ($p < .05$). The probiotics group showed greater improvement in mental flexibility test and stress score than the placebo group ($p < .05$). Contrary to placebo, probiotics significantly increased serum BDNF level ($p < .05$). Notably, the gut microbes significantly shifted by probiotics (*Eubacterium* and Clostridiales) showed significant negative correlation with serum BDNF level only in the probiotics group ($R_S = -0.37$, $R_S = -0.39$, $p < .05$). In conclusion, probiotics promote mental flexibility and alleviate stress in healthy older adults, along with causing changes in gut microbiota. These results provide evidence supporting health-promoting properties of probiotics as a part of healthy diet in the older adults.

Keywords: Probiotics, RCT, Gut microbiota, Cognitive function, Mood status, Healthy older adults

Aging is characterized by progressive decline in biological functions of the organism (1). The functions of the central nervous system also change during normal aging, leading to age-associated cognitive decline and mood disorders that are common and major health issues among older adults (1). Most industrialized countries are facing a rapid increase in the proportion of older adults considered to be in the danger zone of neurological diseases (1,2). Beyond the increasing risk of health issues, the critical social problems such as high economic burden and low growth potential of an aging society have ensued (2). Therefore, development of efficient preventative and therapeutic strategies targeting neurodegenerative disorders should be considered as a public health priority to promote healthy aging in the global population.

The gut microbiota, a collection of microorganisms found in the gastrointestinal tract, has pivotal roles in anatomical, physiological, and immunological host functions (3,4). The gut microbiota

undergoes a significant transition in its composition and function during aging and these alterations can affect health and age-related diseases (5,6). Based on a series of studies, it is now becoming evident that maintaining gut microbial balance during aging is imperative for healthy late life (7). Recently, the emerging concept of gut–brain axis, referring to a bidirectional relationship between gut and brain, has linked gut microbiota to age-related neurodegenerative diseases, such as Alzheimer's disease, and mood disorders including depression and anxiety (8–12). The interplay between gut and brain involves a complex network of endocrinological, immunological, and neural mediators, which has been considered as a critical target for the manipulation of brain health and neurodegenerative diseases (13–15).

Diet is one of the critical lifestyle factors for physical and mental well-being throughout the life span, including later life (16,17). A growing body of evidence suggests that dietary components or nutrients affect various biological functions including brain activity (10,16,18–20). Therefore, research is actively focusing on the emerging concept of brain health preservation through dietary interventions. Probiotics, as part of a healthy diet, have received increasing attention for their potential to regulate brain health via the microbiota–gut–brain axis (9,21). Probiotic bacteria have been shown to affect intestinal microbial dynamics and homeostasis, and influence the physiology of the intestine and distal organs, including the brain (22). However, most of the current evidence comes from animal experiments, and it is crucial yet challenging to assess whether such findings can be translated to humans. Thus, it is critical to validate the clinical properties and effects of probiotics on human gut and brain health, particularly focusing on independently living older individuals, which can be majorly affected by cognitive and mental disorders. Therefore, we conducted a randomized, double-blind, placebo-controlled, multicenter trial to test our hypothesis that probiotic consumption has beneficial effects on intestinal health, and contributes to ameliorate cognitive and mental impairment in the older adults.

Materials and Methods

Study Design

The study was a randomized, double-blind, placebo-controlled, multicenter clinical trial examining the effects of probiotics consumption on intestinal and brain health in elders over the age of 65, conducted at Seoul National University (Seoul, Republic of Korea) and Bundang Jesaeng Hospital (Seongnam, Republic of Korea) from March 2018 to March 2019. The study included a 2-week wash-out period and a 12-week intervention period. During a 2-week wash-out phase, eligible participants were instructed to refrain from dietary supplements including probiotics and other dietary supplements. Participants were then randomly assigned to one of the two following groups: Placebo or Probiotics group. During the intervention period, participants consumed their assigned products twice a day for 12 consecutive weeks. They visited the clinic at baseline (Week 0), Week 4, Week 8, and Week 12 for a compliance check and blood and fecal samples were collected at each visit; and they conducted neuropsychological test at baseline, Week 4 and Week 12. Participants were asked not to change their usual dietary habits and health-related behaviors during the period of intervention. They were asked to record treatment intake, and unusual events such as the use of medication and experiencing adverse events in a daily manner in order to check the adherence to the study. This work is registered with CRiS (Clinical Research Information Service; http://cris.nih.go.kr; https://cris.nih.go.kr/cris/search/search_result_st01_en.jsp?seq=14020&ltype=&rtype=. Registration ID: KCT0003929).

Participants

Participants were recruited from communities in Seoul and Seongnam in the Republic of Korea. Recruitment flyer was posted at Gwanak-gu Community Health Center (Seoul, Republic of Korea), Seoul W Internal Medicine Clinic (Seoul, Republic of Korea) and Bundang Jesaeng Hospital (Seongnam, Republic of Korea). Candidates were invited to an onsite screening, which includes interviews asking about health history, health-related behavior, and dietary habits. Assessment of physical and cognitive functional status was conducted using activities of daily living, instrumental activities of daily living, and Mini-Mental State Examination (MMSE) by experienced research staff.

Criteria for eligibility

Eligible subjects had to be over 65 years old and to consent to be randomly assigned and refrain from consuming any other dietary supplements, which include other probiotics, yogurts with live, active cultures or supplements, and immune-enhancing supplements, during the period of the study. We excluded participants with the use of antibiotics, anti-inflammatory medications, gastrointestinal medicine within the past 3 months; and with regular intake of probiotics within the past 3 months. Participants who are incapable of living independently based on activities of daily living and instrumental activities of daily living score were excluded. A total of 107 candidates entered for screening and a total of 63 subjects enrolled for the study. This study was approved and monitored by the Institutional Review Board of Seoul National University (IRB No. 1801/002-015) and Bundang Jesaeng Hospital (IRB No. IMCN18-01), and written informed consent was obtained from all participants.

Study Capsules

Participants were provided with either placebo or probiotics. For the probiotics, participants were asked to consume two capsules after the meal in the morning and evening, which made a total of four capsules (a total of 1×10^9 colony-forming unit of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI in soybean oil) to be taken per day. For the placebo, each capsule contained 500 mg of soybean oil only. Treatment products were not able to distinguish by package, color, taste, and smell in order to maintain treatment allocation concealed from participants and study staff. Test products were provided by Bifido Inc. (Seoul, Republic of Korea).

Randomization

Study coordinator who was not involved in the study generated a random sequence using GraphPad Prism (version 6.05; GraphPad Software, San Diego, CA) and the random number was stratified by sex with 1:1 allocation. The allocation sequence was concealed from the researchers and details of the allocated group were given on color code containing the sequential number which was prepared by product provider. Independent study coordinator dispensed either placebo or probiotics capsules according to a computer-generated randomized sequence.

Blinding

All participants, study coordinators, and researchers were blinded throughout the entire study. The study was unblinded after all statistical analyses were completed.

Sample Collection

Twelve-hour fasting blood samples and stool samples were collected at each visit (baseline, 4th, 8th, and 12th week). Blood samples were collected into serum separating tube and ethylenediaminetetraacetic acid-coated tubes for serum and plasma isolation, respectively. Serum and plasma samples were aliquoted and immediately stored at −80°C for later analysis. For stool sample collection, we provided a stool collection tube that contains DNA stabilizing preservative reagent (Norgen Biotek, Thorold, ON, Canada). We instructed participants, following manufacturer's instructions, to collect fecal samples into the tubes and mix gently until the stool sample is completely submerged into the preservative. Participants were instructed to collect stool samples within the 48-hour period before visiting; the tubes were kept tightly sealed and stored at room temperature (15–25°C) until they were shipped. After the samples were shipped to the laboratory, aliquots of 180~200 mg of stool samples were immediately stored at −80°C until later analysis.

Outcome Assessments

The primary outcomes include results from cognitive function and mood tests at the end of the experiment. The secondary outcomes were gut microbial composition and anthropometric assessments measured at each visit; and neuronal biochemistry marker from the blood (brain-derived neurotrophic factor [BDNF]) at the end of the experiment.

Anthropometric measures

Body weight and height were measured at each visit using weight scales and stadiometers. BMI was calculated as weight in kilograms divided by height in meters squared.

Evaluation of intestinal health

Participants completed a general health questionnaire that asks about improvements in bowel habits at 4th, 8th, and 12th week, respectively. The questionnaire measures 10 bowel habits, asking whether there were improvements in the following parameters in the last 4 weeks: overall bowel health; frequency of defecation; amount of defecation; feeling of incomplete evacuation; stool odor; abdominal cramping; bowel sounds; number of gas passage; abdominal distention; and frequency of diarrhea. Participants responded with a 5-point scale that ranges from 1 ("not at all") to 5 ("very much"); and the higher scores indicate that there was improvement in each parameter.

Gut microbiota analysis

Genomic DNA extraction

Total bacterial DNA was isolated from stool by using the QIAamp fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with the following additional steps. Extracted genomic DNA was confirmed via gel electrophoresis and was quantified by spectrophotometer NanoDrop ND-2000 (Thermo Scientific, Waltham, MA).

Amplification of 16S rRNA gene and sequencing

Hypervariable regions (V3-V4) of 16S ribosomal ribonucleic acid (rRNA) gene were amplified using barcoded universal primers for each sample. Polymerase chain reaction (PCR) was carried out by using BioFact F-Star taq DNA polymerase (BioFACT, Seoul, Republic of Korea). Briefly, a final volume of 50 μL of PCR reaction contained about 20 ng of DNA template, 5 μL of 10× Taq buffer (20 mM Mg^{2+}), 1 μL of 10 mM dNTP mix, 2 μL of forward and reverse barcoded primers (10 pmol/μL), and 0.25 μL of DNA polymerase. PCR reactions were amplified using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The PCR program was as follows: initial for 5 minutes hold at 94°C, followed by 28 cycles of denaturation (30 seconds, 95°C), annealing (30 seconds, 60°C), and extension (30 seconds, 72°C), with a final extension step (10 minutes, 72°C) followed by holding at 4°C. The PCR product was confirmed by using 1% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA). The amplified products were purified with PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, CA) and quantified by the Qubit 2.0 fluorometer (Invitrogen). The size of library was assessed by BioAnalyzer (Agilent Technologies, Santa Clara, CA). The amplicons were pooled and sequenced with an Illumina MiSeq sequencing system (Illumina, San Diego, CA).

Bioinformatic analysis of sequencing data

Microbial sequences were processed using QIIME2 (version 2019.1) (23). Briefly, sequences were denoised to remove the sequences with low-quality score and chimeras via DADA2. Then, denoised sequences were clustered into operational taxonomic units (OTUs) and OTU representative sequences were aligned based on SILVA database (version 132) at 99% sequence identity with scikit-learn Naive Bayes-based machine-learning classifier. A phylogenetic tree was generated using MAFFT and FastTree method for diversity analyses. Downstream analyses on alpha diversity were carried out to measure dissimilarities in richness and evenness of microbial community. Comparisons of relative abundance between groups were performed to identify the differential features across the samples.

Evaluation of cognitive function and mood status

The Korean version of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD-K) was used to measure cognitive function. The CERAD-K, a validated measure for the screening of Alzheimer's disease, assesses cognitive function including 11 tests measuring domains of language function, memory function, visuospatial processing function, and attention and executive function (24).

A validated 20-item self-reported questionnaire was used to ask the level of stress in a category of burn-out, depression, and anger during the past 1 month (25). Participants responded with a 5-point scale that ranges from 1 ("never") to 5 ("very often"). Total scores were calculated, and higher scores mean higher level of stress. The quality of life (QoL) was measured with the Satisfaction With Life Scale (SWLS), a validated subjected report of global life satisfaction (26). It consists of five items with a 7-point scale that ranges from 1 ("not at all") to 7 ("very much"). Responses were summed and higher scores indicate higher QoL. The Korean version of Geriatric Depression Scale (GDS-K) was used to evaluate the level of depression. The GDS-K is a 30-item self-reported questionnaire which is a validated instrument for the diagnosis of clinical depression (27). Each question was answered with binary responses ("yes" or "no") and scored as either 0 or 1 point. The cumulative score is calculated, and the higher score means the higher level of depression. The Positive Affect and Negative Affect Schedule (PANAS) is a validated self-report instrument in the assessment of positive and negative affect (28). The PANAS is comprised of two 10-item scales which

measure both positive and negative affect, respectively. Each item is assessed with 5-point scale of 1 ("not at all") to 5 ("very much"). The summed scores from each positive and negative affect indicate the level positive and negative affect, respectively.

Serum biochemical markers

Serum BDNF level was measured using BDNF DuoSet ELISA kit (DY248; R&D Systems, Wiesbaden-Nordenstadt, Germany) and DuoSet Ancillary Reagent kit 2 (DY008; R&D Systems) according to the manufacturer's instructions. Briefly, sample or standard was added to a plate coated with capture antibody and the plate was incubated for 2 hours at room temperature. After washing the plate sufficiently, detection antibody was added to the plate and the plate was incubated for 2 hours at room temperature. Streptavidin conjugated to horseradish peroxidase was added to each well and the plate was incubated for 20 minutes at room temperature. Then substrate solution was added to each well for 20 minutes of incubation at room temperature following sufficient washing with wash buffer, and the plate was ready for determining the optical density at 450 nm wavelength using a microplate reader (SpectraMax iD3, Molecular Devices, Austria).

Statistical Analysis

Sample size

To detect a significant change in cognitive function with a two-sided 5% significance level and a power of 80%, a sample size of 32 was determined, given a 20% of dropout rate.

Analysis plan

The normality assumption and homogeneity of variance were tested by Kolmogrov–Smrinov test for study variables. For the comparison analysis of variables at the baseline between groups, we used independent *t*-test, χ^2 tests, or Fisher's Exact Tests. To compare the difference between groups at each time point and delta value between the visits (Δ(Week 4–Week 0), Δ(Week 8–Week 0), and Δ(Week 12–Week 0)) between the two groups, we performed unpaired *t*-test, Mann–Whitney *U* test or generalized linear model (GLM). To compare the difference between baseline and the data from end point (Week 12), we used paired *t*-test or Wilcoxon signed rank test. To detect the difference between groups over the visits, we used a mixed-model analysis of variance (ANOVA) or Friedman test as a nonparametric alternative to the repeated measures ANOVA. Correlations were assessed by Spearman rank correlation analysis. Correction for multiple testing was performed based on the false discovery rate or Bonferroni correction. The $p < .05$ and false discovery rate <0.05 were considered statistically significant in all statistical analyses. All statistical analyses were conducted using Partek (version 6.6; Partek, Saint Louis, MI), SPSS (version 25.0; SPSS Inc., Chicago, IL), or GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA).

Results

General Characteristics of Participants at Baseline

A total of 107 volunteers were screened for eligibility and 63 subjects were enrolled for the study (Supplementary Figure 1). Sixty-three participants were randomized, with 31 and 32 subjects in the placebo and probiotics group, respectively. Fifty-three individuals completed the study and 10 participants withdrew the consent and discontinued the study, and no clinically relevant adverse events were reported during the intervention. When comparing characteristics of participants who withdrew ($N = 10$) and those who completed the trial ($N = 53$), there were no significant differences (Supplementary Table 1). Therefore, we confirmed that randomization was successful. In all analyses, we included data from participants who completed the intervention. Demographic and clinical characteristics at baseline are summarized in Supplementary Table 2. Average age was 72.00 and 71.11 years in the placebo and probiotics group, respectively, with no significant difference ($p = .4538$). The ratio of male to female and BMI did not significantly differ between two groups. Socioeconomic characteristics, including educational level, marital status and type of household, and other health-related characteristics such as cigarette use, alcohol use, physical activity, and self-evaluated health status, were not different between the placebo and probiotics group. In addition, cognitive functions and depression scores, determined by MMSE and Geriatric Depression Scale (GDS-K), were not different between two groups at the baseline.

Probiotic Supplementation Beneficially Influenced Intestinal Health and Gut Microbial Communities

To assess the effect of probiotics on intestinal health, participants filled questionnaire at 4th week, 8th week, and 12th week, respectively, asking whether there were improvements in bowel habits in the last 4 weeks. Bowel habits such as frequency and amount of defecation; feeling of incomplete evacuation; stool odor; number of gas passage; bowel sounds; and abdominal distention were not significantly improved both in the placebo and probiotics group during the intervention period (data not shown); however, scores in frequency of gas passage and abdominal distention showed significant improvements in the probiotics group compared with the placebo (3.44 ± 0.19 vs 2.77 ± 0.21; 3.15 ± 0.22 vs 2.46 ± 0.22, respectively; $p < .05$, Figure 1A and B).

In order to address whether the improvement was driven by any changes in the intestinal bacterial communities, gut microbiome profiling analysis was performed in all participants. Bacterial genomic DNAs from stool samples collected at baseline, 4th week, 8th week, and 12th week were sequenced using 16S rRNA sequencing technology. After preprocessing of bacterial sequences for quality control as described in the Methods, we obtained a total of 10,273,269 raw reads and average reads of 80,260 per sample. To examine the effect of probiotics consumption on gut microbial diversity, we calculated Pielou's evenness index, Faith's phylogenetic diversity,

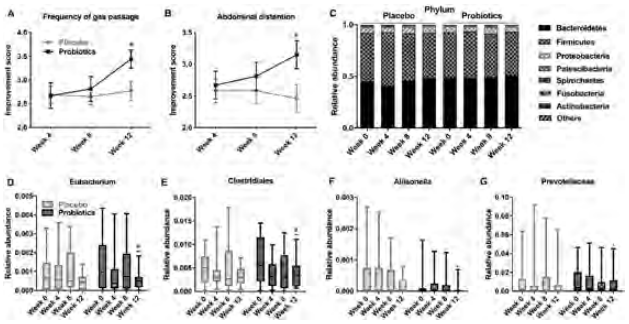

Figure 1. Beneficial influence of probiotic supplementation on intestinal health and gut microbiota. (**A,B**) Improvement scores in frequency of gas passage and abdominal distention measured at each visit are shown. Data are presented as mean (SEM). (**C**) Relative abundance of the gut microbiota at the phylum level and (**D–G**) at the genus level was measured throughout the intervention. Data are presented as mean (min-max). $^{\#}p < .05$ based on the Mann–Whitney *U* test; $^{**}p < .005$, $^{*}p < .05$ based on a post hoc analysis of Friedman test.

observed OTUs, and Shannon's diversity index. We found no significant changes in the diversity both in the placebo and probiotics group during the intervention period (Supplementary Figure 2A–D). Further, we compared the relative abundance of OTUs and specific bacterial taxa at the different phylogenetic levels. Microbial composition at OTU level showed no significant differences during the intervention both in the placebo and probiotics group (Supplementary Figure 2E). At the phylum level, no significant changes in relative abundance were detected during the intervention both in the placebo and probiotics group (Figure 1C). However, at the genus level, we found significant changes in the gut microbial composition in the probiotics group and no changes in the control group (Figure 1D–G). The relative abundances of *Eubacterium, Allisonella,* Clostridiales, and Prevotellaceae gradually changed during the intervention, and significantly decreased at Week 12 in the probiotics group ($p < .05$).

Probiotic Supplementation Improved Brain Function and Increased Peripheral BDNF Levels

To evaluate the impacts of probiotic supplementation on cognitive function, each participant was tested by the CERAD-K, a validated cognitive test battery that scores language, memory, visuo-spatial processing, and attention/executive functions. The assessment was performed at baseline, Week 4, and Week 12 (Table 1). The changes at the fourth week from baseline in the probiotics group were not different from those in the placebo group for all the domains of the cognitive assessment; however, the changes at Week 12 from baseline in the scores of mental flexibility test were significantly different between placebo and probiotics group (Table 2). Interestingly, mental flexibility showed a significant improvement at Week 12 in the probiotics group compared with the placebo group ($p < .05$, Figure 2A). In addition, study subjects filled series of questionnaires to evaluate the impact of probiotics on mood status including quality of life, stress, depression, and positive and negative affect. The 12-week consumption of probiotics did not change the scores of quality of life, GDS-K, and PANAS; however, it did affect the stress score (Table 2). While the stress score was increased in the placebo group (1.38 ± 0.86), it was dramatically decreased in the probiotics group (−2.85 ± 1.16; $p < .05$, Figure 2B).

The observations that probiotic supplementation improved the cognitive function and mental stress prompted us to determine the level of BDNF in blood. BDNF is a neurotrophic factor known to be crucial for learning, memory function, and stress. In contrast to the placebo group (−3.32 ± 2.35), serum BDNF level was significantly increased at Week 12 in the probiotics group (3.68 ± 2.69; $p < .05$, Figure 3A). In addition, to address the question of whether changes in intestinal bacterial communities be related to the serum level of BDNF, we conducted correlation analysis between the relative abundance of each genera and the level of BDNF. It is of interest that *Eubacterium* and Clostridiales showed a significant negative correlation with the level of serum BDNF only in the probiotics group ($R_S = -0.37$ and $R_S = -0.39$; $p < .05$, Figure 3B). These findings suggest that reduction in the relative abundances of *Eubacterium* and Clostridiales in the gut driven by probiotic supplementation closely related to the increase in the serum BDNF, thereby improving brain functions.

Discussion

In the present study, we conducted a randomized, double-blind, placebo-controlled, multicenter trial to address the impact of

Table 1. Cognitive Function Before and After the Intervention

	Placebo ($N = 26$)				Probiotics ($N = 27$)					
	Week 0	Week 4	Week 12	[a]*p*-value	Week 0	Week 4	Week 12	[a]*p*-value	[b]*p* for Δ(Week 4–Week 0)	[b]*p* for Δ(Week 12–Week 0)
Language function										
Verbal fluency	14.96 (4.05)	16.42 (4.59)*	16.88 (4.55)	.01	14.44 (4.48)	15.67 (5.17)	15.41 (4.17)	.40	.86	.39
Boston naming test	11.69 (2.19)	12.19 (2.26)	12.23 (2.23)	.28	12.15 (1.56)	12.70 1.49)	12.96 (1.34)**	<.005	.70	.23
Memory function										
Word list encoding	18.92 (4.42)	20.77 (3.63)	22.23 (4.74)**	<.001	18.26 (2.81)	21.33 (3.10)**	22.22 (3.79)**	<.001	.12	.47
Word list recall	6.38 (1.92)	7.27 (1.78)	7.54 (1.92)*	.01	6.19 (1.82)	6.85 (1.88)	7.52 (1.65)**	<.005	.58	.68
Word list savings	83.83 (18.87)	94.24 (14.47)	92.21 (16.50)	.13	84.68 (23.06)	83.44 (19.30)†	89.30 (13.27)	.18	.15	.71
Word list recognition	8.77 (1.77)	9.27 (1.25)	9.23 (1.53)	.09	9.22 (0.97)	9.37 (0.74)	9.63 (0.63)	.30	.38	.88
Constructional praxis recall	8.15 (2.81)	8.88 (2.57)	9.23 (2.30)	.02	7.93 (3.05)	8.89 (2.39)	9.52 (1.72)*	<.001	.90	.42
Visuo-spatial processing function										
Constructional praxis	10.04 (1.43)	10.00 (1.60)	10.27 (1.34)	.36	10.00 (1.64)	10.33 (1.44)	10.52 (1.01)	.31	.55	.74
Attention and executive function										
Trail making test A	61.88 (26.82)	49.73 (17.99)*	47.35 (15.91)**	<.001	47.33 (18.78)†	46.22 (21.47)	44.41 (21.62)	.06	.36	.21
Trail making test B	189.69 (82.28)	164.65 (77.22)	161.19 (78.26)	.15	172.59 (86.95)	148.26 (67.32)	131.11 (58.44)*	.01	.97	.39
Mental flexibility	2.15 (0.97)	2.46 (1.54)	2.52 (1.68)	.48	2.72 (1.56)	2.30 (0.96)	2.08 (0.85)	.10	.06	.03
Digit span test	13.35 (5.18)	14.23 (4.23)	13.65 (4.54)	.59	13.41 (4.48)	15.33 (3.81)**	14.59 (3.88)*	<.005	.23	.16

Note: Data are presented as mean (*SD*).
[a]*p*-value from Friedman test; [b]*p*-value from the Mann–Whitney *U* test or GLM analysis (after adjusting for baseline value) for between-group analysis for the comparison of delta value; **$p < .005$, *$p < .05$ based on a post hoc analysis of Friedman test; †$p < .05$ based on the Mann–Whitney *U* test for between-group analysis at each time point.

Table 2. Mood Status Before and After the Intervention

	Placebo (N = 26)				Probiotics (N = 27)					
	Week 0	Week 4	Week 12	[a]p-value	Week 0	Week 4	Week 12	[a]p-value	[b]p for Δ(Week 4–Week 0)	[b]p for Δ(Week 1 2–Week 0)
QoL	22.88 (7.26)	21.38 (5.45)	21.58 (6.68)	.07	20.74 (5.85)	20.33 (6.77)	20.89 (6.93)	.38	.12	.18
Stress	31.12 (12.24)	31.73 (13.17)	32.50 (11.46)	.37	31.74 (11.77)	29.33 (8.61)	28.89 (7.77)	.10	.10	.04
Depression	6.31 (5.87)	6.23 (6.16)	6.15 (5.72)	.74	7.59 (4.61)	6.96 (5.36)	6.41 (5.35)	.12	.27	.39
Positive affect	28.46 (7.89)	26.08 (7.20)*	25.58 (6.25)*	.09	28.59 (6.69)	27.52 (6.35)	28.41 (8.28)	.86	.59	.12
Negative affect	16.62 (6.54)	15.54 (5.41)	15.54 (5.27)	.39	14.78 (4.73)	14.67 (4.54)	14.04 (4.75)	.66	.88	.69

Note: Data are presented as mean (*SD*). QoL = Quality of life.
[a]p-value from Friedman test; [b]p-value from the Mann–Whitney U test for between-group analysis for the comparison of delta; $^{**}p < .005$, $^{*}p < .05$ based on a post hoc analysis of Friedman test; $^{\dagger}p < .05$ based on the Mann–Whitney U test for between-group analysis at each time point.

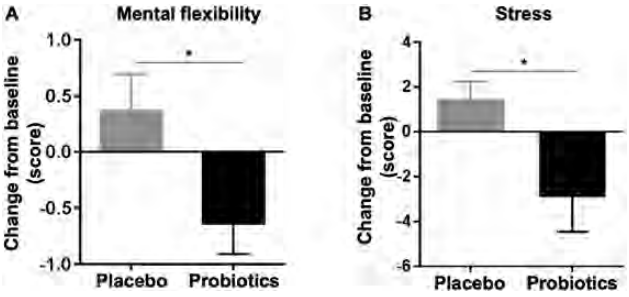

Figure 2. Improved cognitive and mental functioning after probiotic supplementation. (**A**) Change from baseline of cognitive performance score in the mental flexibility test is shown. Reduction in the performance score of mental flexibility indicates improved attention and executive function. (**B**) Change from baseline of stress level is shown. Reduction in the change indicates a reduced level of mental stress. Data are presented as mean (SEM). $^{*}p < .05$ based on the Mann–Whitney U test for the comparison analysis of changes from baseline between the two groups.

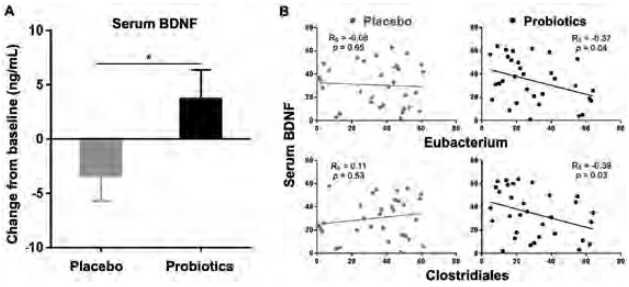

Figure 3. Elevated level of serum BDNF after probiotic supplementation. (**A**) Change from baseline of serum BDNF is shown. Data are presented as mean (SEM). $^{*}p < .05$ for time × treatment from a mixed-model analysis of variance. (**B**) Scatter diagrams with regression lines show the relationship between relative abundance of shifted gut bacteria after probiotic supplementation and the level of serum BDNF. Measurements were rank-normalized and plotted separately for the placebo and probiotics group. Correlation coefficient (R_s) and p-values based on Spearman rank correlation analysis. BDNF = Brain-derived neurotrophic factor.

probiotics on intestinal health and how they contribute to ameliorating cognitive and mental decline in the older adults. Our findings demonstrate that probiotics have system-wide effects on the gut–brain axis in healthy community-dwelling older adults by promoting cognitive and mental health and changing the gut microbial composition.

Emerging evidence has suggested that probiotics have considerable impacts on various cerebral functions through the regulation of the gut–brain axis, but the current studies are mainly focused on patients with mild cognitive impairment, Alzheimer's disease, and major depressive disorder (29–32). However, while neurodegenerative disorders and psychological distress are a common threat to well-being in old age, nutritional intervention to prevent or delay age-associated decline in brain function in the general older population is still underexplored. In fact, there is only one report on the effects of probiotic consumption in healthy older adults, showing that milk fermented by *Lactobacillus helveticus* IDCC3801 improved cognitive functions in healthy older adults (33). However, the sample size was too small and the criteria for the study participants did not represent the general population of older adults. Therefore, the critical need for clinical studies in the general population has been raised. To the best of our knowledge, this is the first well-controlled

clinical study demonstrating system-wide effects of probiotics on the gut–brain axis, which encompasses the large-scale analysis of the gut microbiota and multiple aspects of brain functions in healthy older population.

Randomized controlled trials (RCTs) are very challenging for several issues but the most rigorous method, which provides the most reliable evidence for clinical practice; however, there are few RCTs specifically designed for older adults because it is difficult to recruit older people, particularly community-dwelling older adults (34–36). Therefore, older adults have been excluded from clinical trials and most studies focused on older group of patients (36). In addition, it is relatively hard to follow up and contact older people during a trial which increases the risk of dropout and reduces compliance (36,37). Despite these challenges, in the present study, participant compliance was good as the average rate of compliance to intervention was 96.5%, with a dropout rate of 15.9% only. Moreover, it is important to note that the present study recruited older adults without diseases, not focusing on good responders to a treatment effect such as patients with neurological disorders, which makes our findings more applicable as a generalized health care strategy in community-dwelling older population.

In the probiotics group, the gut microbial composition shifted gradually, and the most relevant change was the reduction in the abundance of bacteria that cause inflammation including *Eubacterium*, *Allisonella*, and Prevotellaceae. It has been identified that *Eubacterium* and Prevotellaceae species, which were significantly reduced after probiotic consumption, are proinflammatory microbiota associated with autoimmune disease and chronic intestinal inflammation in mice (38,39). Of note, the genus *Allisonella*, whose abundance was significantly reduced in the probiotics group, produces histamine, a biogenic monoamine inducing proinflammatory response both centrally and systemically (40). Moreover, in patients with Alzheimer's disease, elevated levels of histamine stimulate neuroinflammation via induction of low-grade systemic inflammation (40). Therefore, these findings may parallel our hypothesis that probiotic supplementation in the older adults may negatively affect inflammaging, a characteristic of chronic low-grade inflammatory status in older adults, via the modulation of microbial composition. However, further studies are required to assess whether the probiotic supplementation affects immunological mechanisms.

The findings of the present study suggest that interaction between the gut microbiota and the central nervous system may underlie the improvements in cognitive and cerebral functioning upon probiotic supplementation and explain the concomitant changes in peripheral neuromodulators. BDNF, a neurotrophic factor vital for synaptic formation, plasticity, and neuroimmune responses, has long been studied to assess its critical role in learning, memory formation, and affective disorders (41,42). Previously, the influence of diet and nutrition on BDNF has been explored; and serum BDNF has been shown to be increased in response to dietary supplements in humans. For example, a 1-week of oral consumption of α-linolenic acid increased the level of serum BDNF in healthy young adults (43). Also, a 6-week supplementation with natural extracts rich in flavonoids and polyphenolic compounds enhanced serum BDNF levels in physically active men (44). In the present study, it was notable that the beneficial impact of a 12-week probiotic intervention on serum BDNF levels was evident in older population. More recently, BDNF has emerged as a pivotal link in the gut–brain axis (41,42). Several studies demonstrated that gut dysbiosis correlates with reduced expression of BDNF, which alters cognitive function and triggers anxiety-like behavior in germ-free animals (45,46), supporting a role of BDNF in the gut–brain axis. Interestingly, we observed that the relative abundance of significantly shifted gut microbes correlated with the level of serum BDNF in the probiotics group only. This indicates that administration of probiotics may affect the interaction between the gut microbiome and the host BDNF, thereby improving brain functions. Overall, the evidence from this study shows that the shifts in microbial community mirrored changes in the cognitive and mental scores.

Several mechanisms could explain the interaction between changes in abundance of commensal bacteria and brain function observed in the probiotics group. First, it is plausible that the production of neurotransmitters, such as γ-Aminobutyric acid (GABA), dopamine, acetylcholine, serotonin, by commensal bacteria, and neurochemicals including BDNF, may directly or indirectly modulate cognition and mood status (47). As shown in a previous study, probiotic administration influences GABA receptor throughout the brain, with reduced stress-induced anxiety- and depression-like behaviors in rodents (48). Moreover, inflammation-mediated pathways might initiate the pathogenesis of neurodegeneration via the microbiota–gut–brain axis. With respect to inflammaging during normal aging, chronic low-grade inflammation in older adults may affect neuroinflammation by modulating glial cells, which stimulates cognitive impairment (47). One of the routes to translate systemic inflammatory signals into the brain is stimulation of microglia by peripheral cytokines that cross the blood–brain barrier, leading to a proinflammatory status in the brain and dysregulation of neurological processes (47). Additionally, the immunomodulatory roles of circulating immune cells in neuroplasticity also affect the expression of BDNF (49,50). Therefore, it is plausible that mitigation of inflammaging in older adults with probiotic intervention might positively impact on cognitive and mental functions via the modulation of BDNF signaling. Further studies are needed to clearly demonstrate the effect of probiotics on inflammatory status and gut microbiome at the functional level.

The present study is not without limitations. First, direct evidence of improvement in peripheral and cerebral inflammation by probiotic consumption is lacking, which might be the crucial interface linking the gut–brain axis in the present study. Therefore, further mechanistic studies might be needed to elucidate the role of probiotic supplementation by finding biomarkers to link the axis. Second, although cognitive functions were evaluated by validated neuropsychological assessment battery tasks taking at least 60 minutes per subject by a professionally trained panel, psychological assessments of mood status were based on the participants' self-reporting; therefore, possible recall bias may exist. Additionally, although our results indicated that the benefit of 3 months duration of probiotic intervention was evident, there were no significant changes in some of the cognitive functions in the neuropsychological assessment battery in which we assume that the study duration was not enough to monitor the improvements. Therefore, further studies are required with a longer period of intervention. Despite these limitations, this is the first study examining the effects of probiotic supplementation on brain functions in community-dwelling older adults. In conclusion, our study showed that probiotic supplementation is beneficial for improving cognitive and mental health in community-dwelling healthy older adults with changes in gut microbial composition. These results provide evidence that probiotics have health-promoting properties as part of a healthy diet in the general population of independently living older adults.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

Funding

This research was funded by the National Research Foundation of Korea (NRF-2015S1A5A2A03050006), the Promoting Regional specialized Industry (R0004140), the Ministry of Trade, Industry and Energy (MOTIE), and Korea Institute for Advancement of Technology (KIAT), Republic of Korea.

Authors' Contributions

C.K., H.B., and D.S. designed the research; C.K., L.C., and M.S. conducted the research; C.K. performed bioinformatic and statistical analyses and analyzed the data; W.C. conducted the statistical analysis; and C.K. and D.S. wrote the manuscript. The authors thank S.J. for his assistance in recruitment of participants; D.S. had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of Interest

None reported.

References

1. Mattson MP, Arumugam TV. Hallmarks of brain aging: adaptive and pathological modification by metabolic states. *Cell Metab*. 2018;27:1176–1199. doi: 10.1016/j.cmet.2018.05.011
2. World Health Organization. *World Report on Ageing and Health*. Geneva: World Health Organization; 2015.
3. Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet*. 2003;361:512–519. doi: 10.1016/S0140-6736(03)12489-0
4. Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol*. 2016;16:341–352. doi: 10.1038/nri.2016.42
5. O'Toole PW, Jeffery IB. Gut microbiota and aging. *Science*. 2015;350:1214–1215. doi: 10.1126/science.aac8469
6. Vaiserman AM, Koliada AK, Marotta F. Gut microbiota: a player in aging and a target for anti-aging intervention. *Ageing Res Rev*. 2017;35:36–45. doi: 10.1016/j.arr.2017.01.001
7. Clark RI, Walker DW. Role of gut microbiota in aging-related health decline: insights from invertebrate models. *Cell Mol Life Sci*. 2018;75:93–101. doi: 10.1007/s00018-017-2671-1
8. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. *Front Physiol*. 2011;2:94. doi: 10.3389/fphys.2011.00094
9. Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The central nervous system and the gut microbiome. *Cell*. 2016;167:915–932. doi: 10.1016/j.cell.2016.10.027
10. Sandhu KV, Sherwin E, Schellekens H, Stanton C, Dinan TG, Cryan JF. Feeding the microbiota-gut-brain axis: diet, microbiome, and neuropsychiatry. *Transl Res*. 2017;179:223–244. doi: 10.1016/j.trsl.2016.10.002
11. Foster JA, McVey Neufeld KA. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci*. 2013;36:305–312. doi: 10.1016/j.tins.2013.01.005
12. Seo D-O, Holtzman DM. Gut microbiota: from the forgotten organ to a potential key player in the pathology of Alzheimer's disease. *J Gerontol A Biol Sci Med Sci*. 2019;18:glz262. doi:10.1093/gerona/glz262
13. Forsythe P, Bienenstock J, Kunze WA. Vagal pathways for microbiome-brain-gut axis communication. *Adv Exp Med Biol*. 2014;817:115–133. doi: 10.1007/978-1-4939-0897-4_5
14. Dinan TG, Cryan JF. The microbiome-gut-brain axis in health and disease. *Gastroenterol Clin North Am*. 2017;46:77–89. doi: 10.1016/j.gtc.2016.09.007
15. Sun Y, Baptista LC, Roberts LM, et al. The gut microbiome as a therapeutic target for cognitive impairment. *J Gerontol A Biol Sci Med Sci*. 2019. doi:10.1093/gerona/glz281
16. Moore K, Hughes CF, Ward M, Hoey L, McNulty H. Diet, nutrition and the ageing brain: current evidence and new directions. *Proc Nutr Soc*. 2018;77:152–163. doi: 10.1017/S0029665117004177
17. Grønning K, Espnes GA, Nguyen C, et al. Psychological distress in elderly people is associated with diet, wellbeing, health status, social support and physical functioning- a HUNT3 study. *BMC Geriatr*. 2018;18:205. doi: 10.1186/s12877-018-0891-3
18. O'Neil A, Quirk SE, Housden S, et al. Relationship between diet and mental health in children and adolescents: a systematic review. *Am J Public Health*. 2014;104:e31–e42. doi: 10.2105/AJPH.2014.302110
19. Oddy WH, Allen KL, Trapp GSA, et al. Dietary patterns, body mass index and inflammation: pathways to depression and mental health problems in adolescents. *Brain Behav Immun*. 2018;69:428–439. doi: 10.1016/j.bbi.2018.01.002
20. Opie RS, Itsiopoulos C, Parletta N, et al. Dietary recommendations for the prevention of depression. *Nutr Neurosci*. 2017;20:161–171. doi: 10.1179/1476830515Y.0000000043
21. Kim CS, Shin DM. Probiotic food consumption is associated with lower severity and prevalence of depression: a nationwide cross-sectional study. *Nutrition*. 2019;63-64:169–174. doi: 10.1016/j.nut.2019.02.007
22. Blander JM, Longman RS, Iliev ID, Sonnenberg GF, Artis D. Regulation of inflammation by microbiota interactions with the host. *Nat Immunol*. 2017;18:851–860. doi: 10.1038/ni.3780
23. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010;7:335–336. doi: 10.1038/nmeth.f.303
24. Lee JH, Lee KU, Lee DY, et al. Development of the Korean Version of the Consortium to Establish a Registry for Alzheimer's Disease Assessment Packet (CERAD-K) clinical and neuropsychological assessment batteries. *J Gerontol B Psychol Sci Soc Sci*. 2002;57:P47–P53. doi:10.1093/geronb/57.1.p47
25. Lee E, Shin H, Yang Y, Cho J, Ahn K, Kim S. Development of the stress questionnaire for KNHANES: report of scientific study service. Seoul: *Korea Centers for Disease Control and Prevention*; 2010.
26. Diener E, Emmons RA, Larsen RJ, Griffin S. The satisfaction with life scale. *J Pers Assess*. 1985;49:71–75. doi: 10.1207/s15327752jpa4901_13
27. Bae JN, Cho MJ. Development of the Korean version of the Geriatric Depression Scale and its short form among elderly psychiatric patients. *J Psychosom Res*. 2004;57:297–305. doi: 10.1016/j.jpsychores.2004.01.004
28. Lee H-H, Kim E-J, Lee M-K. A validation study of Korea positive and negative affect schedule: the PANAS scales. *Korean J Clin Psychol*. 2003;22:935–946.
29. Akbari E, Asemi Z, Daneshvar Kakhaki R, et al. Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's disease: a randomized, double-blind and controlled trial. *Front Aging Neurosci*. 2016;8:256. doi: 10.3389/fnagi.2016.00256
30. Rudzki L, Ostrowska L, Pawlak D, et al. Probiotic *Lactobacillus plantarum* 299v decreases kynurenine concentration and improves cognitive functions in patients with major depression: a double-blind, randomized, placebo controlled study. *Psychoneuroendocrinology*. 2019;100:213–222. doi: 10.1016/j.psyneuen.2018.10.010
31. Kazemi A, Noorbala AA, Azam K, Eskandari MH, Djafarian K. Effect of probiotic and prebiotic vs placebo on psychological outcomes in patients with major depressive disorder: a randomized clinical trial. *Clin Nutr*. 2019;38:522–528. doi: 10.1016/j.clnu.2018.04.010
32. Akkasheh G, Kashani-Poor Z, Tajabadi-Ebrahimi M, et al. Clinical and metabolic response to probiotic administration in patients with major depressive disorder: a randomized, double-blind, placebo-controlled trial. *Nutrition*. 2016;32:315–320. doi: 10.1016/j.nut.2015.09.003
33. Chung Y-C, Jin H-M, Cui Y, et al. Fermented milk of *Lactobacillus helveticus* IDCC3801 improves cognitive functioning during cognitive fatigue tests in healthy older adults. *J. Funct. Foods*. 2014;10:465–474. doi: 10.1016/j.jff.2014.07.007
34. Kammerer K, Falk K, Herzog A, Fuchs J. How to reach 'hard-to-reach'older people for research: the TIBaR model of recruitment. *Survey Methods: Insights from the Field (SMIF)*. 2019. Retrieved from https://surveyinsights.org/?p=11822. doi: 10.25646/6315
35. Broekhuizen K, Pothof A, de Craen AJ, Mooijaart SP. Characteristics of randomized controlled trials designed for elderly: a systematic review. *PLoS One*. 2015;10:e0126709. doi: 10.1371/journal.pone.0126709

36. Clegg A, Relton C, Young J, Witham M. Improving recruitment of older people to clinical trials: use of the cohort multiple randomised controlled trial design. *Age Ageing*. 2015;44:547–550. doi: 10.1093/ageing/afv044
37. Ungar A, Marchionni N. *Cardiac Management in the Frail Elderly Patient and the Oldest Old*. Cham: Springer; 2017.
38. Palm NW, de Zoete MR, Cullen TW, et al. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell*. 2014;158:1000–1010. doi: 10.1016/j.cell.2014.08.006
39. Zhou C, Zhao H, Xiao X-y, et al. Metagenomic profiling of the pro-inflammatory gut microbiota in ankylosing spondylitis. *J Autoimmun*. 2020;107:102360.
40. Westfall S, Lomis N, Kahouli I, Dia SY, Singh SP, Prakash S. Microbiome, probiotics and neurodegenerative diseases: deciphering the gut brain axis. *Cell Mol Life Sci*. 2017;74:3769–3787. doi: 10.1007/s00018-017-2550-9
41. Licinio J, Wong M-. Brain-derived neurotrophic factor (BDNF) in stress and affective disorders. *Mol Psychiatry*. 2002;7:519. doi: 10.1038/sj.mp.4001211
42. Bauer KC, Huus KE, Finlay BB. Microbes and the mind: emerging hallmarks of the gut microbiota-brain axis. *Cell Microbiol*. 2016;18:632–644. doi: 10.1111/cmi.12585
43. Hadjighassem M, Kamalidehghan B, Shekarriz N, et al. Oral consumption of α-linolenic acid increases serum BDNF levels in healthy adult humans. *Nutr J*. 2015;14:20. doi: 10.1186/s12937-015-0012-5
44. Sadowska-Krępa E, Kłapcińska B, Pokora I, Domaszewski P, Kempa K, Podgórski T. Effects of six-week Ginkgo biloba supplementation on aerobic performance, blood pro/antioxidant balance, and serum brain-derived neurotrophic factor in physically active men. *Nutrients*. 2017;9:803. doi: 10.3390/nu9080803
45. Diaz Heijtz R, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci USA* 2011;108:3047–3052. doi: 10.1073/pnas.1010529108
46. Gareau MG, Wine E, Rodrigues DM, et al. Bacterial infection causes stress-induced memory dysfunction in mice. *Gut*. 2011;60:307–317. doi: 10.1136/gut.2009.202515
47. Di Benedetto S, Müller L, Wenger E, Düzel S, Pawelec G. Contribution of neuroinflammation and immunity to brain aging and the mitigating effects of physical and cognitive interventions. *Neurosci Biobehav Rev*. 2017;75:114–128. doi: 10.1016/j.neubiorev.2017.01.044
48. Bravo JA, Forsythe P, Chew MV, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* 2011;108:16050–16055. doi: 10.1073/pnas.1102999108
49. Gibney SM, McGuinness B, Prendergast C, Harkin A, Connor TJ. Poly I:C-induced activation of the immune response is accompanied by depression and anxiety-like behaviours, kynurenine pathway activation and reduced BDNF expression. *Brain Behav Immun*. 2013;28:170–181. doi: 10.1016/j.bbi.2012.11.010
50. Cortese GP, Barrientos RM, Maier SF, Patterson SL. Aging and a peripheral immune challenge interact to reduce mature brain-derived neurotrophic factor and activation of TrkB, PLCγ1, and ERK in hippocampal synaptoneurosomes. *J Neurosci*. 2011;31:4274–4279. doi: 10.1523/JNEUROSCI.5818-10.2011