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Immunomodulatory Effects of *Lactobacillus* and *Bifidobacterium* on Both Murine and Human Mitogen-Activated T Cells

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Key Words
Probiotics · *Lactobacillus* · *Bifidobacterium* · T cell

Abstract
Background: Beneficial effects of probiotics have been reported for patients with allergic diseases and intestinal disorders. There is increasing interest in studying the role of different strains or combined probiotic administration on immunoregulation. In this study, we investigated whether probiotics modulate the immune response through regulating T cell proliferation and differentiation. ***Methods:*** We examined the effect of probiotic I (a combination of *Lactobacillus acidophilus* and *Bifidobacterium bifidus*) and probiotic II (a combination of *L. acidophilus* and *B. infantis*) on cell survival and proliferation, the progression of the cell cycle, and the production of Th1/Th2 cytokines by mitogen-stimulated murine spleen cells and human peripheral blood mononuclear cells (PBMCs). ***Results:*** Our experimental results showed that high concentrations ($\geq 1 \times 10^6$ CFU/ml) of probiotic I or II inhibited mitogen-induced cell proliferation and arrested the cell cycle at the G0/G1 stage in both mitogen-stimulated spleen cells and PBMCs. In the results of low concentrations ($<1 \times 10^6$ CFU/ml), probiotic I or II enhanced the production of IFN-γ but inhibited the production of IL-4. Our results indicated that high concentrations of probiotic I or II treatment could attenuate mitogen-induced overactive immune responses. On the other hand, low concentrations of probiotic I or II treatment could promote a shift in the Th1/Th2 balance toward Th1-skewed immunity. ***Conclusion:*** Dose selection is an important issue for probiotic studies. Our results indicated that probiotics have beneficial effects on regulating T cell-mediated immune responses by attenuating mitogen-induced overactive immune responses and promoting Th1 immune responses.

Introduction

Probiotics are live nonpathogenic bacteria that confer health benefits beyond their nutritional value [1]. The therapeutic potential of probiotics has been demonstrated mainly in experimental colitis as well as in human inflammatory bowel diseases [2]. Normal intestinal flora is important for health and, kept in balance, it crucially influences the normal structural and functional development of the mucosal immune system [3]. Microaerophilic lactobacilli and anaerobiotic bifidobacteria are important members of the human indigenous flora of the large intestine and have beneficial effects in humans [4]. Our previ-

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ous report indicated that oral probiotics *Lactobacillus acidophilus* and *Bifidobacterium infantis* reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants [5]. The possible mechanisms by which probiotics may protect from necrotizing enterocolitis include competitive exclusion of potential pathogens [6], inhibition of the growth of pathogens [7, 8], and modification of the host immune response to microbial products [9, 10].

The therapeutic benefits of *L. acidophilus* and bifidobacteria are mediated by (i) the prevention of intestinal infections and diarrheal diseases, (ii) the enhancement of immunity, (iii) the prevention of colon cancer, (iv) the improvement of lactose utilization, and (v) stabilization of the gut mucosal barrier, among other factors [11]. Lactobacilli are frequently used as probiotics to promote human health due to their effects on potentiating the immune response and preventing intestinal infection, among many others [12–14]. Previous studies have reported that *L. acidophilus* induces the production of TNF-α, IL-6, and IL-10 by human peripheral blood mononuclear cells (PBMCs) [15], enhances the activation and maturation of mouse and human dendritic cells [16, 17], and induces the apoptosis of antigen-stimulated T cells [18]. On the other hand, *Bifidobacterium* is a dominant genus of infants' fecal flora that represent up to 90% of the total gut microflora in breast-fed babies [19] and up to 15% of that in adults [20]. Some strains of the genus *Bifidobacterium* exhibit anti-inflammatory properties [21–23], increase intestinal IgA secretion [24], and induce dendritic cell maturation [25].

There is increasing interest in studying the role of different strains of probiotics on proinflammatory and anti-inflammatory cytokine secretion from macrophages. These cytokines are important in regulating local inflammatory responses [1, 21, 26]. In Crohn's disease, the potent proinflammatory cytokine TNF-α seems to play a pivotal role in the pathogenesis of altered mucosal immune function [27, 28]. Several observations lead to the notion that enhanced secretion of Th1-type cytokines, such as IL-2 and IFN-γ, and TNF-α, acts as a key factor in the pathogenesis of Crohn's disease [29, 30]. Additionally, IFN-γ mainly augments cellular immunities and exhibits antitumor responses, and it inhibits the production of Th2-type cytokines such as IL-4. It is well known that helper T cells play a major role in the regulation of mucosal immune responses. Accordingly, the effects of probiotics on the functions of T cells could help to understand the possible role of probiotics in human health. The aim of this study was to investigate whether probiotics modulate the immune response through regulating T cell proliferation and differentiation.

Materials and Methods

Mice

BALB/c mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and maintained in the Animal Center of China Medical University (Taichung, Taiwan). The animal room was kept on a 12-hour light and dark cycle with a constant temperature and humidity. All mice were bled at 8 weeks of age. All procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, USA).

Reagents

Concanavalin A (ConA), phytohemagglutinin (PHA), phorbol myristate acetate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ionomycin, propidium iodide, Triton X-100, and EDTA were purchased from Sigma Chemical (St. Louis, Mo., USA). RPMI-1640 medium, Hank's balanced salt solution, penicillin, streptomycin, L-glutamine, and fetal calf serum were purchased from Gibco BRL (Grand Island, N.Y., USA). Ficoll-Hypaque density gradient was purchased from Amersham Biosciences (Uppsala, Sweden). The MRS broth, Rogosa SL agar, and raffinose-*Bifidobacterium* agar were purchased from Difco Laboratories (Detroit, Mich., USA). ELISA kits for the detection of human and mouse IFN-γ and IL-4 were purchased from BD Pharmingen (San Diego, Calif., USA). For intracellular staining, brefeldin A, fixation buffer, permeabilization buffer, FITC-labeled anti-human IFN-γ mAb, PE-labeled anti-human IL-4 mAb, FITC-labeled anti-mouse IFN-γ mAb, and PE-labeled anti-mouse IL-4 mAb were purchased from eBioscience (San Diego, Calif., USA).

Bacterial Culture

L. acidophilus (10^9 CFU NCDO 1748, obtained from the National Collection of Dairy Organisms, Reading, UK), *B. bifidum* (10^9 CFU NCDO 1453, obtained from the National Collection of Dairy Organisms), and *B. infantis* (strain ATCC 15697, obtained from the American Type Culture Collection, Rockville, Md., USA) were activated in MRS broth. The MRS broth was subcultured in Rogosa SL agar for differential isolated *L. acidophilus* and it was subcultured in raffinose-*Bifidobacterium* agar for differential isolated *B. bifidum* and *B. infantis*. All plates were incubated at 37°C for 48 h under anaerobic conditions. Probiotic I (contains *L. acidophilus* and *B. bifidus*) and probiotic II (contains *L. acidophilus* and *B. infantis*) were used in the following experiments.

Cell Culture

Animals were sacrificed by cervical spine dislocation. The spleen was removed and crushed into a single cell suspension, and red blood cells were lysed by Tris-buffered ammonium chloride before washing 3 times with Hank's balanced salt solution [31]. Human whole blood was collected from healthy volunteers and the PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation as described previously [31]. Cell numbers were determined using a hemocytometer, and viabilities were assessed via the trypan blue dye exclusion method. Cells were seeded at a density of 2×10^6 cells/ml and incubated at 37°C in humidified 5% CO_2/95% air to allow macrophages to adhere. Two hours later, the nonadherent cells were collected and incubated with medium containing various concentrations of test compounds. The mitogenic response to plant lectins (ConA or PHA) is conventionally

used to measure cell-mediated immunity in mammals [32, 33]. Both ConA and PHA mitogens stimulate T lymphocytes by indirectly cross-linking the T cell receptor complex [34]. Control cells were grown under identical conditions but were not exposed to the test compounds or mitogen. All culture materials were purchased from BD Biosciences (San Jose, Calif., USA); it had been clearly indicated that these were endotoxin free.

MTT Assay

Human PBMCs and murine spleen cells were seeded at a density of 1×10^5 cells/ml in 96-well U-bottom plates and incubated with different concentrations of probiotic I or II in the presence or absence of ConA or PHA for 2 days at 37°C in humidified 5% CO_2/95% air. In addition, we seeded the untreated cells at a range of 1.25×10^4–4×10^5 cells/ml in 96-well U-bottom plates for constructing a standard curve which was used to calculate the cell number at the end of the incubation period. After 48 h, cells were incubated with basal medium containing 0.5 mg/ml MTT in a CO_2 incubator at 37°C for 4 h. The plates were centrifuged (5 min at 500 *g*) and supernatants were removed. Hydrochloric acid (0.04 N) in isopropanol (100 μl) was added to each well and the absorbance was measured at 570 nm (reference wavelength 650 nm) with a microplate reader (VersaMax; Molecular Devices, Sunnyvale, Calif., USA).

Cytokine Assay

Cells were incubated with different concentrations of probiotic I, probiotic II, *L. acidophilus*, *B. bifidus*, or *B. infantis* in the presence or absence of ConA or PHA for 2 days. The supernatants were then harvested and stored at –80°C until analyzed by ELISA.

Cell Cycle Analysis

Human PBMCs and murine spleen cells were stimulated with 5 μg/ml ConA or PHA and different concentrations of probiotic I, probiotic II, *L. acidophilus*, *B. bifidus*, or *B. infantis* for 3 days and cell cycle analysis was performed using the method described previously [35]. Briefly, cells were washed with PBS and stained with 20 μg/ml propidium iodide in 0.1% Triton X-100, and 0.1 mM EDTA. Cell suspensions were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif., USA). The percentages of cell cycle distribution in the G0/G1, S, and G2/M phases were determined using MODFIT software (Becton Dickinson).

Intracellular Staining

Human PBMCs and murine spleen cells were incubated with different concentrations of probiotic I or II in the presence or absence of ConA or PHA for 2 days at 37°C in humidified 5% CO_2/95% air. At the end of the incubation period, cells were stimulated with 10^{-7} M phorbol myristate acetate plus 1 μg/ml ionomycin for 6 h. Brefeldin A (10 μg/ml) was added during the last 2 h of culture. For intracellular staining, cells were fixed with fixation buffer for 30 min and permeabilized with permeabilization buffer for 30 min following the manufacturer's instructions (eBiosciences). Cells were stained with FITC-labeled anti-IFN-γ and PE-labeled anti-IL-4 mAb (eBiosciences) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

Statistical Analysis

All experimental data were shown as means ± SD and accompanied by the number of independent experiments. For in vitro data, statistical analysis was performed using Student's t test, and differences were regarded as statistically significant for p values of less than 5% ($p < 0.05$), 1% ($p < 0.01$), and 0.1% ($p < 0.001$).

Results

The Effect of Probiotics on Cell Survival and Proliferation in Both Murine Spleen Cells and Human PBMCs

We examined whether probiotic I or II affect cell survival in both murine spleen cells and human PBMCs. Murine spleen cells and human PBMCs were treated with various dosages (4×10^6, 2×10^6, 1×10^6, and 0.2×10^6 CFU/ml) of probiotic I or II in the presence or absence of ConA or PHA for 2 days. Cell survival was examined by MTT assay. The experimental results showed that probiotic I or II did not affect cell survival under these treatment dosages in either murine or human mitogen-activated T cells (fig. 1). However, probiotic I or II inhibited both human and murine mitogen-activated T cell proliferation at concentrations of $\geq 1 \times 10^6$ CFU/ml (fig. 1). In order to examine whether probiotic I or II could divide and proliferate in our culture conditions, we performed an MTT assay to determine the viability of probiotic I or II alone. Our results of the MTT assay showed that the OD values of the probiotic I or II culture were indistinguishable from the background (data not shown). This also indicated that probiotics I and II did not divide and proliferate in our culture conditions.

The Effect of Probiotics on the Cell Cycle Distribution of Mitogen-Activated Spleen Cells

We examined the effects of probiotic I or II on the cell cycle distribution of spleen cells. Murine spleen cells were treated with various dosages (4×10^6, 2×10^6, 1×10^6, and 0.2×10^6 CFU/ml) of probiotic I or II in the presence or absence of ConA for 2 days. The experimental results showed that probiotic I did not affect the cell cycle distribution of spleen cells (table 1). However, 2×10^6 or higher concentrations of probiotic II could promote the cell cycle progression of spleen cells from G1 to the S phase (table 1). On the other hand, our experimental results showed that treating ConA-stimulated spleen cells with 1×10^6 CFU/ml or higher concentrations of probiotic I or II significantly increased the percentage of cells in the G0/G1 phase and dramatically decreased the cell population in the S phase (table 1). In addition, treating ConA-stimulated spleen cells with probiotic I induced more cells to accumulate in the G0/G1 cell cycle compared to

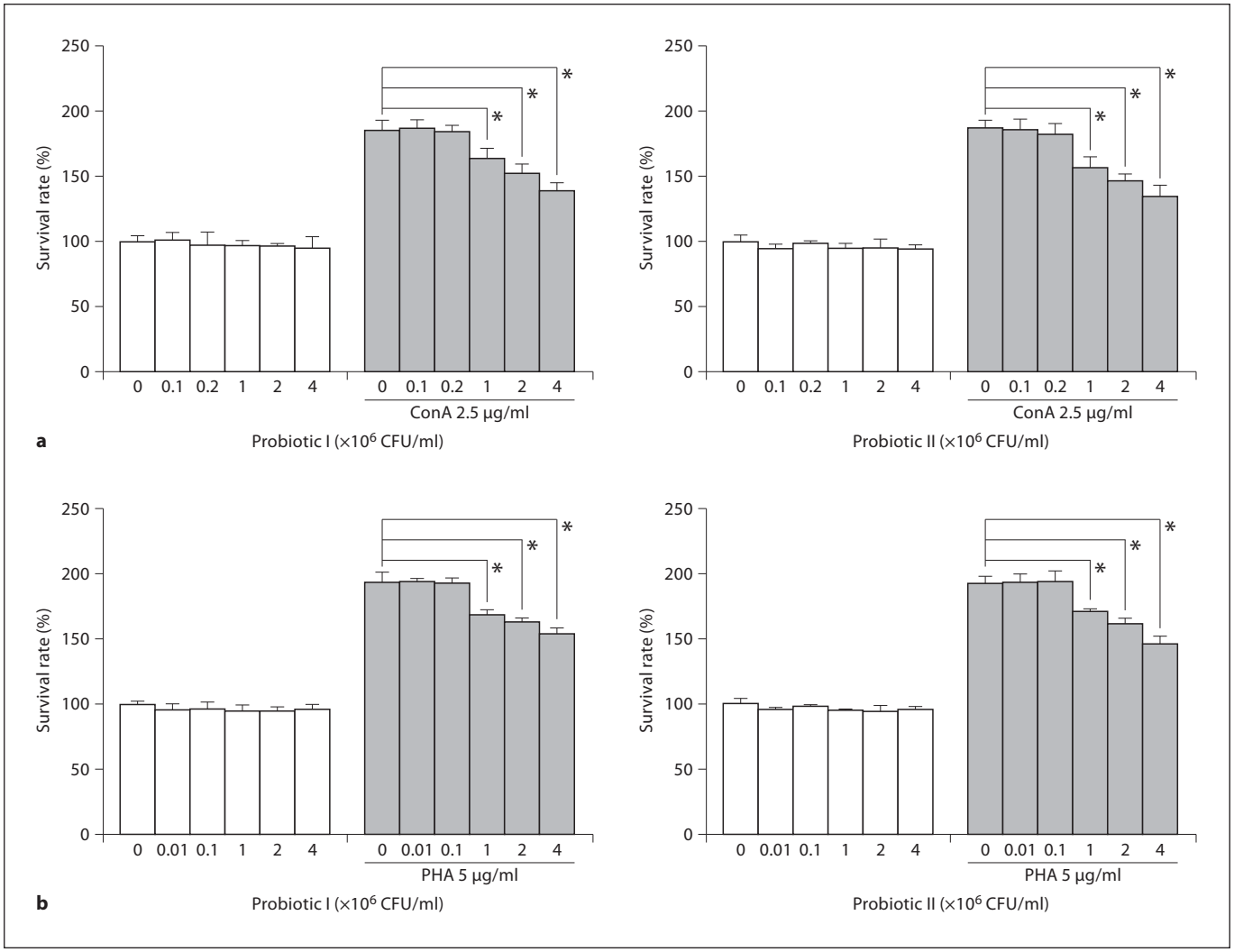

Fig. 1. Effects of probiotics I and II on cell survival in both murine and human mitogen-activated T cells. Human PBMCs and murine spleen cells were treated with various dosages of probiotic I or II in the presence or absence of 2.5 μg/ml ConA or 5 μg/ml PHA for 2 days. Cell survival was examined by MTT assay. The data represent the means ± SD of triplicate experiments. Statistical analysis was performed using Student's t test. * $p < 0.05$.

treatment with probiotic II (table 1). Furthermore, to clarify which probiotics contribute to arresting mitogen-stimulated spleen cells' cell cycle, we studied the individual activity of the bacteria in probiotic I or II. Different amounts of *L. acidophilus*, *B. bifidus*, or *B. infantis* were used to treat spleen cells in the presence of ConA. The results showed that these 3 strains had similar activities in arresting the mitogen-activated cell cycle in the G0/G1 stage in the presence of 2×10^6 CFU/ml or higher concentrations (for details, see online supplementary table 1, www.karger.com/doi/10.1159/000322350).

The Effect of Probiotics on Th1/Th2 Cytokine Production by ConA-Stimulated Spleen Cells

While investigating the effects of probiotic I and probiotic II on Th1- and Th2-type cytokine production, we detected the Th1-type cytokine, IFN-γ, and Th2-type cytokine, IL-4, produced by ConA-stimulated spleen cells in the presence of probiotic I or II. The experimental results showed that treating ConA-stimulated spleen cells with 1×10^6, 0.2×10^6, or 0.1×10^6 CFU/ml of probiotic I or II significantly promoted the production of IFN-γ compared to treatment with ConA alone (fig. 2a).

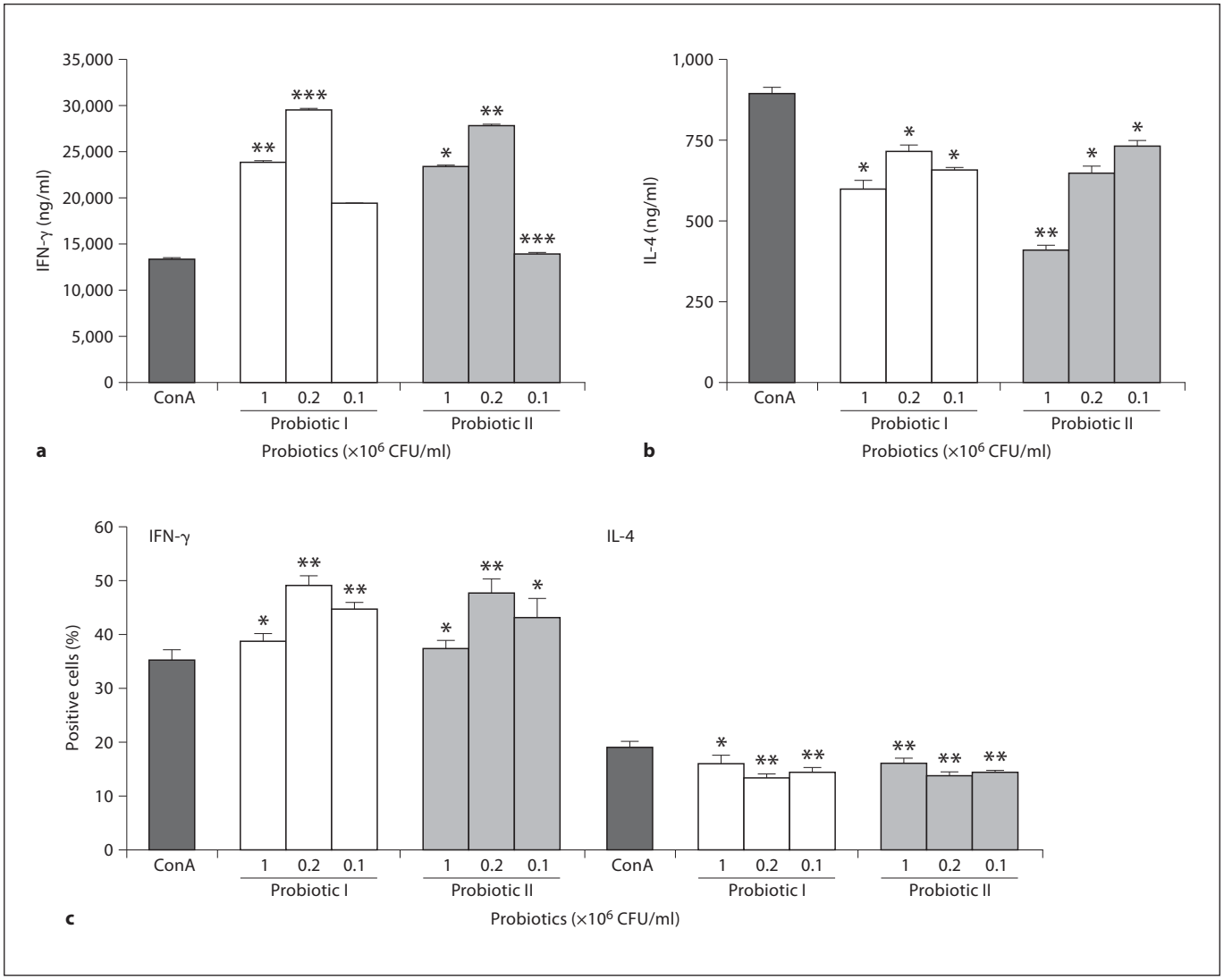

Fig. 2. Effects of probiotics I and II on Th1/Th2 cytokine production by mitogen-stimulated spleen cells. Spleen cells were stimulated with 2.5 μg/ml ConA and different concentrations of probiotic I or II for 2 days. Supernatants were collected and the levels of IFN-γ (**a**) and IL-4 (**b**) were determined by ELISA. **c** Analysis of intracellular cytokine production by ConA-stimulated spleen cells treated with probiotic I or II. The data represent the means ± SD of triplicate cultures. Statistical analysis was performed using Student's t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In contrast, treating cells with 1×10^6, 0.2×10^6, or 0.1×10^6 CFU/ml of probiotic I or II decreased the production of IL-4 by ConA-stimulated spleen cells (fig. 2b). Treating spleen cells with probiotic I or II alone did not affect the production of IFN-γ or IL-4 (data not shown). We further confirmed the cytokine produced by ConA-stimulated spleen cells treated with probiotic I or II using an intracellular staining assay. Our experimental results indicated that treating ConA-stimulated spleen cells with probiotic I or II increases the proportion of IFN-γ-producing cells (fig. 2c). In contrast, treating ConA-stimulated spleen cells with probiotic I or II decreases the proportion of IL-4-producing cells (fig. 2c). Treating spleen cells with probiotic I or II alone did not affect the proportion of IFN-γ- or IL-4-producing cells (data not shown).

Table 1. Effects of probiotic I and probiotic II on the cell cycle distribution of spleen cells

	G0/G1	S	G2/M
Spleen cells only	91.6	7.88	0.52
2.5 μg/ml ConA	54.05	39.67	6.28
Probiotic I			
4×10^6 CFU/ml + 2.5 μg/ml ConA	93.17***	0.02***	6.81
2×10^6 CFU/ml + 2.5 μg/ml ConA	85.53***	0.03***	14.44*
1×10^6 CFU/ml + 2.5 μg/ml ConA	78.26**	4.68***	17.05*
0.2×10^6 CFU/ml + 2.5 μg/ml ConA	52.91	39.99	7.10
4×10^6 CFU/ml	98.22	1.55	0.24
2×10^6 CFU/ml	96.06	3.88	0.06
1×10^6 CFU/ml	93.11	4.08	2.81
0.2×10^6 CFU/ml	91.84	6.10	2.06
Probiotic II			
4×10^6 CFU/ml + 2.5 μg/ml ConA	76.34**	23.64*	0.02**
2×10^6 CFU/ml + 2.5 μg/ml ConA	69.34*	28.84*	1.82**
1×10^6 CFU/ml + 2.5 μg/ml ConA	64.58*	26.84*	8.58
0.2×10^6 CFU/ml + 2.5 μg/ml ConA	57.37	36.59	6.04
4×10^6 CFU/ml	79.15^{+}	20.84^{++}	0.01
2×10^6 CFU/ml	83.93^{+}	13.23^{+}	2.84
1×10^6 CFU/ml	91.04	6.46	2.50
0.2×10^6 CFU/ml	91.75	5.56	2.68

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to cells treated with ConA only. $^{+}$ $p < 0.05$; $^{++}$ $p < 0.01$ compared to cells only.

Table 2. Effects of probiotic I and probiotic II on the cell cycle distribution of PBMCs

	G0/G1	S	G2/M
PBMCs only	96.63	1.21	2.16
5 μg/ml PHA	61.94	24.98	13.08
Probiotic I			
4×10^6 CFU/ml + 5 μg/ml PHA	89.37***	5.11***	5.52**
2×10^6 CFU/ml + 5 μg/ml PHA	80.5**	8.59***	10.91
1×10^6 CFU/ml + 5 μg/ml PHA	73.79**	16.77**	9.43*
0.1×10^6 CFU/ml + 5 μg/ml PHA	69.05*	20.23*	10.72
0.01×10^6 CFU/ml + 5 μg/ml PHA	62.51	21.54	15.95
4×10^6 CFU/ml	97.41	1.05	1.54
2×10^6 CFU/ml	95.25	0.89	3.86
1×10^6 CFU/ml	96.28	1.11	2.61
0.1×10^6 CFU/ml	97.58	1.23	1.19
0.01×10^6 CFU/ml	96.57	1.01	2.42
Probiotic II			
4×10^6 CFU/ml + 5 μg/ml PHA	88.09***	5.64***	6.27**
2×10^6 CFU/ml + 5 μg/ml PHA	81.73**	9.3***	8.97**
1×10^6 CFU/ml + 5 μg/ml PHA	74.31**	15.08**	10.61
0.1×10^6 CFU/ml + 5 μg/ml PHA	66.21	22.29	11.51
0.01×10^6 CFU/ml + 5 μg/ml PHA	63.15	24.89	11.96
4×10^6 CFU/ml	98.25	1.21	0.54
2×10^6 CFU/ml	97.54	1.13	1.33
1×10^6 CFU/ml	97.15	1.06	1.79
0.1×10^6 CFU/ml	96.48	1.05	2.47
0.01×10^6 CFU/ml	96.78	0.99	2.23

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to cells treated with PHA only.

The Effect of Probiotics on the Cell Cycle Distribution of Mitogen-Stimulated PBMCs

We further compared the effects of probiotic I or II on the cell cycle distribution of PBMCs. Human PBMCs were treated with various dosages (4×10^6, 2×10^6, 1×10^6, 0.1×10^6, and 0.01×10^6 CFU/ml) of probiotic I or II in the presence or absence of PHA for 2 days. The experimental results showed that probiotic I or II did not affect the cell cycle distribution of PBMCs (table 2). On the other hand, our experimental results showed that 0.1×10^6 CFU/ml or higher concentrations of probiotic I arrested the PHA-stimulated cell cycle distribution in the G0/G1 stage (table 2). In addition, 1×10^6 CFU/ml or higher concentrations of probiotic II arrested the PHA-stimulated cell cycle distribution in the G0/G1 stage (table 2). Furthermore, the effects of each strain of bacteria of probiotic I or II on PHA-stimulated PBMCs were also examined. We found that 0.1×10^6 CFU/ml or higher concentrations of *L. acidophilus* or *B. bifidus* had similar inhibitory effects on G0/G1 arrest. In addition, 1×10^6 CFU/ml of *B. infantis* arrested the PHA-stimulated PBMCs in the G0/G1 stage (online suppl. table 2).

The Effect of Probiotics on Th1/Th2 Cytokine Production by PHA-Stimulated PBMCs

We further examined the effect of probiotic I and probiotic II on Th1- and Th2-type cytokine production by PHA-stimulated PBMCs. The experimental results showed that 0.1×10^6 or 0.01×10^6 CFU/ml of probiotic I or II promoted the production of IFN-γ by PHA-stimulated PBMCs (fig. 3a). In contrast, 0.1×10^6 and 0.01×10^6 CFU/ml of probiotic I or II decreased the production of IL-4 by PHA-stimulated PBMCs in a dose-dependent manner (fig. 3b). Treating PBMCs with probiotic I or II alone did not affect the production of IFN-γ or IL-4 (data not shown). We further confirmed the cytokine produced by PHA-stimulated PBMCs treated with probiotic I or II using an intracellular staining assay. Our

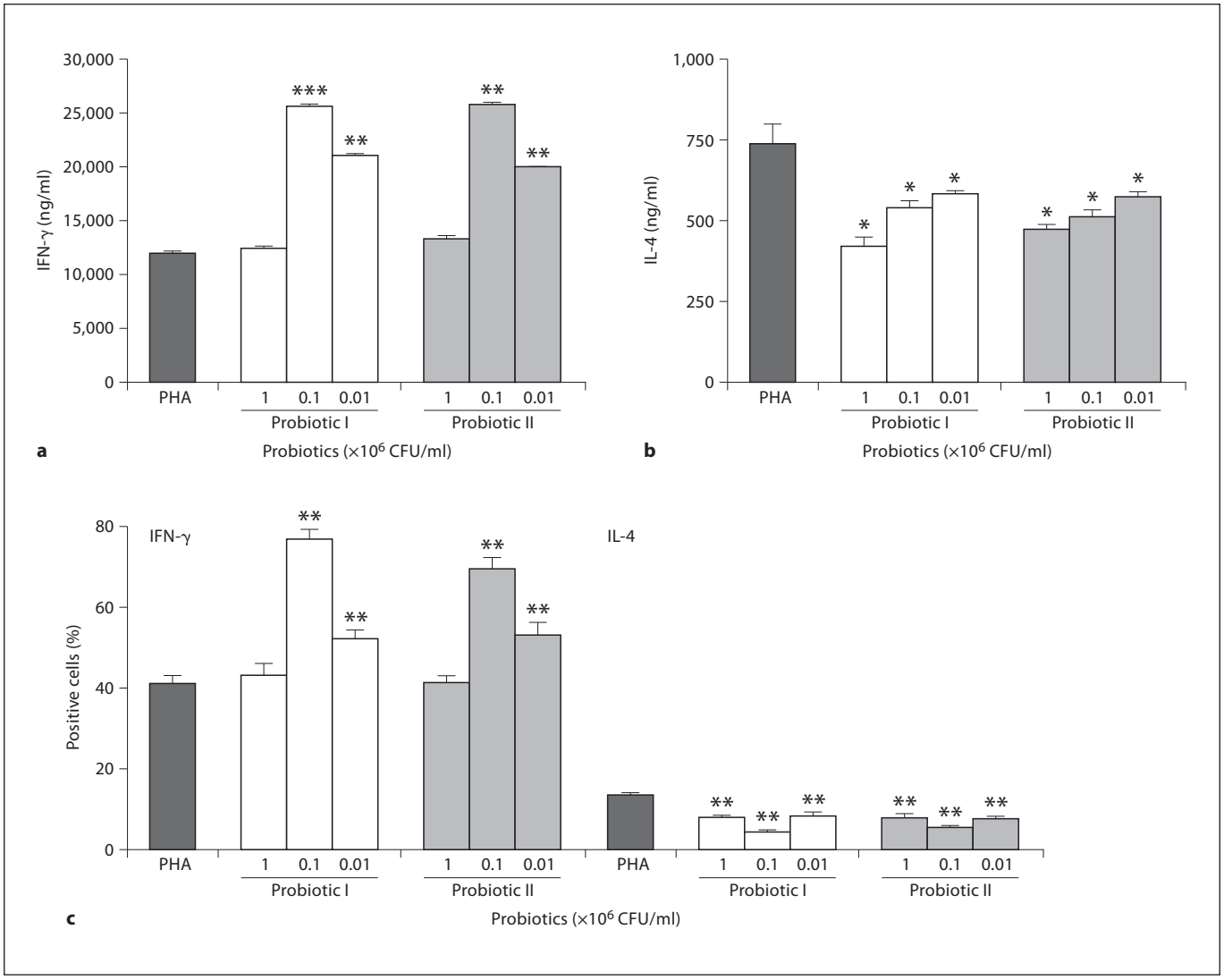

Fig. 3. Effects of probiotics I and II on Th1/Th2 cytokine production by mitogen-stimulated PBMCs. PBMCs were stimulated with 5 μg/ml PHA and different concentrations of probiotic I or II for 2 days. Supernatants were collected and the levels of IFN-γ (**a**) and IL-4 (**b**) were determined by ELISA. **c** Analysis of intracellular cytokine production by PHA-stimulated PBMCs treated with probiotic I or II. The data represent the means ± SD of triplicate cultures. Statistical analysis was performed using Student's t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

experimental results indicated that treating PHA-stimulated PBMCs with probiotic I or II increases the proportion of IFN-γ-producing cells (fig. 3c). In contrast, treating PHA-stimulated PBMCs with probiotic I or II decreased the proportion of IL-4-producing cells (fig. 3c). Treating PBMCs with probiotic I or II alone did not affect the proportion of IFN-γ- or IL-4-producing cells (data not shown).

Discussion

Health claims of probiotics used in functional food and pharmaceutical preparations are based on the capacity of these microorganisms to stimulate the host immune system [36]. Probiotics are gaining interest as alternatives for antibiotics or anti-inflammatory drugs [37]. However, their mode of action in immunoregulation is poorly understood. In the present study, we examined

whether probiotics modulate the immune response through regulating T cell proliferation and differentiation and focused on the effect of combined probiotic administration on the immunomodulatory function.

Our results showed that treating cells with high concentrations ($\geq 1 \times 10^6$ CFU/ml) of probiotic I or II inhibits its mitogen-induced cell proliferation and arrests the cell cycle distribution in the G0/G1 phase in both mitogen-stimulated spleen cells and PBMCs. Stimulation with *L. acidophilus*, *B. bifidus*, or *B. infantis* alone also arrests the cell cycle distribution of mitogen-stimulated spleen cells and PBMCs in the G0/G1 phase. Similar immunomodulatory effects have been reported, maintaining that *L. acidophilus* strain L-92 induces the apoptosis of antigen-stimulated T cells [18]. These results suggest that administration of probiotic I or II could attenuate the mitogen-induced overactive immune response. In addition, our experimental results showed that the combination of probiotic treatments (probiotic I or II) has stronger effects on arresting cell cycle progression in the G0/G1 phase than do treatments with 1 probiotic alone. This finding suggested that combined probiotic treatment may have additive effects on arresting cell cycle progression in the G0/G1 phase in both mitogen-stimulated spleen cells and PBMCs.

Based on our results, 1×10^6 CFU/ml of probiotic I or II had weaker effects on regulating IFN-γ and IL-4 production than $<1 \times 10^6$ CFU/ml of probiotic I or II did. The results of intracellular staining also showed that 1×10^6 CFU/ml of probiotic I or II had weaker effects on intracellular IFN-γ expression. Our results indicated that high concentrations ($\geq 1 \times 10^6$ CFU/ml) of probiotic I or II not only affect mitogen-induced cell proliferation and cell cycle distribution but also reduce the production of IFN-γ in both mitogen-stimulated spleen cells and PBMCs. We suggest that high concentrations ($\geq 1 \times 10^6$ CFU/ml) of probiotic I or II may impair the activity of both mitogen-stimulated spleen cells and PBMCs. A higher IFN-γ/IL-4 ratio was observed when mice ConA-stimulated spleen cells and human PHA-stimulated PBMCs were treated with 0.2×10^6 or 0.1×10^6 CFU/ml of probiotics, respectively. These results suggested that treating cells with probiotic I or probiotic II may lead to a switch in the T cell response from Th2 to Th1. Furthermore, ConA-stimulated spleen cells or human PHA-stimulated PBMCs produced lower amounts of IFN-γ and IL-4 when cells were treated with 1×10^6 CFU/ml of probiotics than when cells were treated with 0.2×10^6 or 0.1×10^6 CFU/ml of probiotics, respectively. These findings implied that lower concentrations of probiotics might be optimal for modulating T cell functions. In this study, we also found that regardless of whether probiotics were pre-heat-treated or not their inhibitory effects on mitogen-activated T cells were similar (data not shown). This suggested that active and inactive probiotics show equal effects on modulating immune cell functions.

Several reports have shown that probiotics skew the Th1/Th2 balance toward Th1 by increasing the production of Th1-type cytokines (IL-12 and IFN-γ) in monocytes, dendritic cells, macrophages, and PBMCs after cells have been incubated with several strains of *Lactobacillus* or *Bifidobacterium* [18, 21, 26, 38]. Furthermore, the effects of *Lactobacillus* or *Bifidobacterium* on the stimulation of Th1 immunity have also been reported in clinical trials [39]. In addition, spleen cells from mice given *L. acidophilus* also produced significantly higher amounts of IFN-γ in response to stimulation with ConA compared to cells from the control mice [40]. Previous studies have indicated that *L. casei* strain Shirota feeding induced a Th1 response rather than a Th2 response [26, 38]. Similarly, we found that either probiotic I or probiotic II promotes a Th1 response in mice and humans. On the other hand, the increased production of IFN-γ suggested that this cytokine may be an important factor in enhancing the cellular immunity and inhibition of cancer cell proliferation that have been observed by many research groups [41–44]. Therefore, these reports may highlight an important immunomodulatory role for commensal bacteria in the gastrointestinal tract.

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References

1 Dotan I, Rachmilewitz D: Probiotics in inflammatory bowel disease: possible mechanisms of action. Curr Opin Gastroenterol 2005;21:426–430.

2 Reiff C, Kelly D: Inflammatory bowel disease, gut bacteria and probiotic therapy. Int J Med Microbiol 2010;300:25–33.

3 Gronlund MM, Lehtonen OP, Eerola E, Kero P: Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J Pediatr Gastroenterol Nutr 1999;28:19–25.

4 Nosova T, Jousimies-Somer H, Jokelainen K, Heine R, Salaspuro M: Acetaldehyde production and metabolism by human indigenous and probiotic *Lactobacillus* and *Bifidobacterium* strains. Alcohol Alcohol 2000;35:561–568.
5 Lin HC, Su BH, Chen AC, Lin TW, Tsai CH, Yeh TF, Oh W: Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. Pediatrics 2005;115:1–4.
6 Reid G, Howard J, Gan BS: Can bacterial interference prevent infection? Trends Microbiol 2001;9:424–428.
7 Coconnier MH, Bernet MF, Chauviere G, Servin AL: Adhering heat-killed human *Lactobacillus acidophilus*, strain LB, inhibits the process of pathogenicity of diarrhoeagenic bacteria in cultured human intestinal cells. J Diarrhoeal Dis Res 1993;11:235–242.
8 Coconnier MH, Bernet MF, Kerneis S, Chauviere G, Fourniat J, Servin AL: Inhibition of adhesion of enteroinvasive pathogens to human intestinal caco-2 cells by *Lactobacillus acidophilus* strain lb decreases bacterial invasion. FEMS Microbiol Lett 1993;110:299–305.
9 Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A: Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci 1995;78:491–497.
10 Schiffrin EJ, Brassart D, Servin AL, Rochat F, Donnet-Hughes A: Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. Am J Clin Nutr 1997;66:515S–520S.
11 Kailasapathy K, Chin J: Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. Immunol Cell Biol 2000;78:80–88.
12 Bloksma N, de Heer E, van Dijk H, Willers JM: Adjuvanticity of lactobacilli. 1. Differential effects of viable and killed bacteria. Clin Exp Immunol 1979;37:367–375.
13 Popova P, Guencheva G, Davidkova G, Bogdanov A, Pacelli E, Opalchenova G, Kutzarova T, Koychev C: Stimulating effect of DEODAN (an oral preparation from *Lactobacillus bulgaricus* 'lb51') on monocytes/macrophages and host resistance to experimental infections. Int J Immunopharmacol 1993;15:25–37.
14 Saavedra JM, Bauman NA, Oung I, Perman JA, Yolken RH: Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet 1994;344:1046–1049.
15 Miettinen M, Vuopio-Varkila J, Varkila K: Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun 1996;64:5403–5405.
16 Drakes M, Blanchard T, Czinn S: Bacterial probiotic modulation of dendritic cells. Infect Immun 2004;72:3299–3309.
17 Zeuthen LH, Christensen HR, Frokiaer H: Lactic acid bacteria inducing a weak interleukin-12 and tumor necrosis factor alpha response in human dendritic cells inhibit strongly stimulating lactic acid bacteria but act synergistically with Gram-negative bacteria. Clin Vaccine Immunol 2006;13:365–375.
18 Kanzato H, Fujiwara S, Ise W, Kaminogawa S, Sato R, Hachimura S: *Lactobacillus acidophilus* strain l-92 induces apoptosis of antigen-stimulated T cells by modulating dendritic cell function. Immunobiology 2008;213:399–408.
19 Fanaro S, Chierici R, Guerrini P, Vigi V: Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl 2003;91:48–55.
20 Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, Cresci A, Silvi S, Orpianesi C, Verdenelli MC, Clavel T, Koebnick C, Zunft HJ, Dore J, Blaut M: Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol 2006;72:1027–1033.
21 Isolauri E, Sutas Y, Kankaanpaa P, Arvilommi H, Salminen S: Probiotics: effects on immunity. Am J Clin Nutr 2001;73:444S–450S.
22 Ouwehand AC, Bergsma N, Parhiala R, Lahtinen S, Gueimonde M, Finne-Soveri H, Strandberg T, Pitkala K, Salminen S: *Bifidobacterium* microbiota and parameters of immune function in elderly subjects. FEMS Immunol Med Microbiol 2008;53:18–25.
23 Okada Y, Tsuzuki Y, Hokari R, Komoto S, Kurihara C, Kawaguchi A, Nagao S, Miura S: Anti-inflammatory effects of the genus *Bifidobacterium* on macrophages by modification of phospho-i kappaB and SOCS gene expression. Int J Exp Pathol 2009;90:131–140.
24 Nakanishi Y, Hosono A, Hiramatsu Y, Kimura T, Nakamura R, Kaminogawa S: Characteristic immune response in Peyer's patch cells induced by oral administration of *Bifidobacterium* components. Cytotechnology 2005;47:69–77.
25 Lopez P, Gueimonde M, Margolles A, Suarez A: Distinct bifidobacterium strains drive different immune responses in vitro. Int J Food Microbiol 2010;138:157–165.
26 Kato I, Tanaka K, Yokokura T: Lactic acid bacterium potently induces the production of interleukin-12 and interferon-gamma by mouse splenocytes. Int J Immunopharmacol 1999;21:121–131.
27 Borruel N, Carol M, Casellas F, Antolin M, de Lara F, Espin E, Naval J, Guarner F, Malagelada JR: Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. Gut 2002;51:659–664.
28 Van Deventer SJ: Tumour necrosis factor and Crohn's disease. Gut 1997;40:443–448.
29 Fiocchi C: Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182–205.
30 Targan SR: Biology of inflammation in Crohn's disease: mechanisms of action of anti-TNF-a therapy. Can J Gastroenterol 2000;14 Suppl C:13C–16C.
31 Fang SH, Hwang LH, Chen DS, Chiang BL: Ribavirin enhancement of hepatitis C virus core antigen-specific type 1 T helper cell response correlates with the increased IL-12 level. J Hepatol 2000;33:791–798.
32 Hovi T, Suni J, Hortling L, Vaheri A: Stimulation of chicken lymphocytes by T- and B-cell mitogens. Cell Immunol 1978;39:70–78.
33 Toivanen P, Toivanen A: Selective activation of chicken T lymphocytes by concanavalin A. J Immunol 1973;111:1602–1603.
34 Abbas AK, Lichtman AH, Pober JS: Cellular and Molecular Immunology, ed 2. Philadelphia, W.B. Saunders, 1994.
35 Rao YK, Fang SH, Tzeng YM: Inhibitory effects of the flavonoids isolated from *Waltheria indica* on the production of NO, TNF-alpha and IL-12 in activated macrophages. Biol Pharm Bull 2005;28:912–915.
36 Perdigon G, Maldonado Galdeano C, Valdez JC, Medici M: Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr 2002;56(suppl 4):S21–S26.
37 Oelschlaeger TA: Mechanisms of probiotic actions – a review. Int J Med Microbiol 2010;300:57–62.
38 Matsuzaki T, Chin J: Modulating immune responses with probiotic bacteria. Immunol Cell Biol 2000;78:67–73.
39 Pohjavuori E, Viljanen M, Korpela R, Kuitunen M, Tiittanen M, Vaarala O, Savilahti E: *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy. J Allergy Clin Immunol 2004;114:131–136.
40 Gill HS, Rutherfurd KJ, Prasad J, Gopal PK: Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (hn001), *Lactobacillus acidophilus* (hn017) and *Bifidobacterium lactis* (hn019). Br J Nutr 2000;83:167–176.
41 Rafter JJ: The role of lactic acid bacteria in colon cancer prevention. Scand J Gastroenterol 1995;30:497–502.
42 Sasaki T, Fukami S, Namioka S: Enhanced resistance of mice to *Escherichia coli* infection induced by administration of peptidoglycan derived from *Bifidobacterium thermophilum*. J Vet Med Sci 1994;56:433–437.
43 Sasaki T, Fukami S, Namioka S: Enhancement of cytotoxic activity of lymphocytes in mice by oral administration of peptidoglycan (PG) derived from *Bifidobacterium thermophilum*. J Vet Med Sci 1994;56:1129–1133.
44 Sekine K, Ohta J, Onishi M, Tatsuki T, Shimokawa Y, Toida T, Kawashima T, Hashimoto Y: Analysis of antitumor properties of effector cells stimulated with a cell wall preparation (WPG) of *Bifidobacterium infantis*. Biol Pharm Bull 1995;18:148–153.