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ARTICLE

Screening of Immune-Active Lactic Acid Bacteria

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Abstract

The purpose of this study was to investigate the effect of lactic acid bacteria (LAB) cell wall extract on the proliferation and cytokine production of immune cells to select suitable probiotics for space food. Ten strains of LAB (*Lactobacillus bulgaricus*, *L. paracasei*, *L. casei*, *L. acidophilus*, *L. plantarum*, *L. delbruekii*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Bifidobacterium breve*, and *Pedicoccus pentosaceus*) were sub-cultured and further cultured for 3 d to reach 7-10 Log colony-forming units (CFU)/mL prior to cell wall extractions. All LAB cell wall extracts failed to inhibit the proliferation of BALB/c mouse splenocytes or mesenteric lymphocytes. Most LAB cell wall extracts except those of *L. plantarum* and *L. delbrueckii* induced the proliferation of both immune cells at tested concentrations. In addition, the production of T_H1 cytokine (IFN-γ) rather than that of T_H2 cytokine (IL-4) was enhanced by LAB cell wall extracts. Of ten LAB extracts, four (from *L. acidophilus*, *L. bulgaricus, L. casei*, and *S. thermophiles*) promoted both cell proliferating and T_H1 cytokine production. These results suggested that these LAB could be used as probiotics to maintain immunity and homeostasis for astronauts in extreme space environment and for general people in normal life.

Keywords: lactic acid bacteria, cell wall extracts, proliferation, T_H1 cytokine IFN-γ, T_H2 cytokine IL-4

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Introduction

Lactic acid bacteria (LAB) have been extensively used to ferment or culture foods from ancient times. LAB include species of *Lactobacillus, Streptococcus, Enterococcus, Lactococcus*, and *Leuconostoc*. They are usually categorized as facultative anaerobes. *Bifidobacterium* is a strict anaerobe (Masood *et al*., 2011). These bacteria are widely distributed in the nature and human digestive tracts.

LAB exert diverse beneficial effects to human. LAB can alleviate peptic ulcer, reduce symptoms of lactose intolerance, lower serum cholesterol, prevent colon cancer, and enhance immunity (Isolauri *et al*., 2004; Masood *et al*., 2011). LAB also can improve intestinal health through producing antimicrobial substances such as bacteriocins with broad or narrow spectrum of antagonistic effect against pathogenic bacteria (Kanmani *et al*., 2013). These properties of LAB have functional advantages as valuable probiotics in health food with diverse therapeutic purposes (Arendt *et al*., 2011; Bhattacharyya, 2009; Logan *et al*., 2003).

Recently, LAB have been planned for space foods to maintain astronauts' health against microbial infection (Ilyin, 2005; National Astronautics and Space Administration, 2014). It has been reported that astronauts undergo a sharp decrease of intestinal LAB such as lactobacilli and bifidobacteria, thus needing greater concentrations of antibiotics to suppress microbial virulence and disregulation of their immune system during a space flight (Crucian and Sams, 2009; Ilyin, 2005; Klaus and Howard, 2006). Although these changes have not been confirmed to affect the susceptibility of astronauts to pathogenic bacteria-induced infections, medical events of astronauts clearly show substantial cases of microbial infections such as conjunctivities and acute respiratory or dental infections (Ball and Evans Jr., 2001). They can cause the bodily deterioration of astronauts during their space mission. As bio-antibiotic agents, LAB can reduce microbial virulence by preventing the growth of pathogenic bacteria. Results using volunteers also have shown that providing LAB as a type of pellets can substantially decrease the quantities of harmful microbial species including *Klebsiella*, Enterobacteriaceae, *Proteus*, and Gram-negative bacteria in their throats and noses (Ilyin, 2005). Therefore, LAB might be used to

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maintain astronauts' capability against microbial infections during space missions.

As a part of the effort to develop probiotics for direct-fed space food, we have screened radio-resistant LAB that could stay active under stressful space condition such as exposure to cosmic ray without changing their beneficial effects to human (Hwang *et al*., 2013). Of 10 commercial LAB tested, *L. acidophilus* has been shown to be the most radio-resistant LAB. In this study, LAB used for the production of fermented milk products were tested for their usability as specialized food in space environment through monitoring their ability in inducing proliferation of immune cells and cytokines such as interferon (IFN)-γ and interleukin (IL)-4.

Material and Methods

LAB strain and media

The following ten LAB stains were purchased from Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea: *L. plantarum* (KCTC3103), *L. casei* (KCTC 3109), *L. delbrueckii* (KCTC13730), *L. delbrueckii* subsp. *bulgaricus* (KCTC3635), *Bifidobacterium breve* (KCTC 3419), *Pediococcus pentosaceus* (KCTC3116), *L. paracasei* (KCTC13169), *Lactococcus lactis* (KCTC2013), *Streptococcus salivarius* subsp. *thermophilus* (KCTC36 58), and *L. acidophilus* (KCTC3140). *S. thermophilus* and *L. lactis* were grown in Brain Heart Infusion (BHI) broth (Difco, USA). The other eight LAB were grown in de Man, Rogosa, and Sharpe (MRS) broth (Difco). Bacteria were stored at -70°C in BHI or MRS broth containing 10% glycerol for future use.

Preparation of LAB cell wall extract

The preparation of LAB was performed according to published method (Hwang *et al*., 2013). Briefly, ten LAB strains were subcultured twice in BHI or MRS broth. One percent subculture LAB suspensions were inoculated and incubated for 3 d at 37°C to reach 7-10 Log CFU/mL. Broth cultures were centrifuged at 5,000 rpm for 20 min at 4°C. The resulting LAB pellets were washed twice with phosphate buffered saline (PBS) (pH 7.4). To obtain cell wall extracts, LAB pellets were suspended in 40 mL distilled water in a 50 mL polypropylene tube and sonicated using Vibracell sonicator (Sonic and Materials, USA) with sonication of 0.2 s and pause of 0.2 s for 10 min. During sonication, cooling was maintained by placing the polypropylene tube in ice-filled bucket. Sonicated LAB samples were centrifuged at 12,000 rpm for 20 min at 4°C. Precipitates were removed. Supernatants were freeze-dried as LAB cell wall extracts and kept in -70°C for further analysis.

Animals

Seven-wk-old female BALB/c mice (body weight 18-20 g) were procured from Orient Co. (Charles River Technology, Korea). For vivarium adaption, mice were housed in polycarbonate cages. They were fed standard diet and water *ad libitum* under controlled temperature condition (22 ± 2°C) with 12 h light and dark cycles for a week prior to experiment.

Preparation of splenocytes and mesenteric lymphocytes from BALB/c mice

Preparation of splenocytes from BALB/c mice was carried out using previously published method (Kim *et al*., 2009). Twelve mice were anesthetized with ether and sacrificed by cervical decapitation. Spleens were collected by dissection and divided into three parts. Each part was placed in Rosewell Park Memorial Institute (RPMI) 1640 medium (Hyclone, USA). Spleen tissues were homogenized with glass homogenizer (Corning Inc., USA). The resulting cell homogenate was centrifuged at 1,000 rpm for 5 min. Cell pellets were gently resuspended in 2 mL of red blood cell lysis buffer (eBioscience Inc., USA) to remove red blood cells. Cell suspension was then mixed with 18 mL of RPMI 1640 medium and centrifuged at 1,000 rpm for 5 min. After centrifugation, cells were washed twice with same medium and resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 unit/mL of penicillin/streptomycin.

Mesenteric lymphocytes were prepared using the same protocol of splenocyte isolation. Briefly, mesenteric lymph nodes were separated from BLAB/C mice by dissection and homogenized with glass homogenizer. Cell homogenate of lymph node was centrifuged and washed twice with RPMI medium without adding red blood cell lysis buffer. Mesenteric lymphocytes were resuspended in RPMI 1640 medium containing 10% FBS and 100 unit/mL of penicillin/streptomycin.

Cell proliferation assay

The suspensions of splenocytes and mesenteric lymphocytes were seeded at 1×10^6 cells/well into 96-well plate and incubated at 5% CO_2 and 37°C for 24 h. Cells were treated with various concentrations (0-125 g/mL) of LAB cell wall extracts and further incubated for 23 h. Ten microliter of MTT (dimethylthiazol tetrazolium bromide;

5 mg/mL in PBS) solution was added into each well and incubated for one hour at 37ºC. The plate was then centrifuged at 2,000 rpm for 10 min. Culture media in each well were removed by aspiration. One hundred microliter of dimethylsulfoxide was added into each well. The plate was incubated at 37°C for 5 min. Absorbance was measured at 595 nm using ELISA reader (Thermo Fischer Scientific, Finland). Cell proliferation was determined as a percentage relative to untreated control cells. As positive controls, concanavalin A (Con A) was used at concentration of 1 g/mL for splenocytes. Phorbol myristate acetate (PMA) and ionomycine (ION) were used in combination at concentration of 50 ng/mL and 0.7 pg/mL, respectively, for mesenteric lymphocytes.

Cytokine production

The culture suspension of splenocytes or mesenteric lymphocytes was used to determine cytokine production using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, USA). Splenocytes or mesenteric lymphocytes incubated in 96-well plate for 24 h were treated with various concentrations (0-125 g/mL) of LAB cell wall extracts at 37°C for 24 h. Culture supernatants were collected. The amounts of IFN-γ and IL-4 released into the medium were measured according to manufacturer's instructions. Absorbance was measured at 450 nm using ELISA reader. The resulting values were converted into concentrations (pg/mL) of INF-γ and IL-4 using standard curves of each standard. As positive controls, Con A, PMA, and ION were used at the same concentrations for cell proliferation assays.

Statistical analysis

Statistical analysis of data was carried out by One Way ANOVA using SPSS 19.0 software. Significantly different means were compared using multiple range Duncan's test and t-test. A P value less than 0.05 was considered statistically significant.

Results and Discussion

Effect of LAB cell wall extracts on cell proliferation

The effect of ten LAB cell wall extracts on cell proliferation of BALB/c mice splenocytes and mesenteric lymphocytes was determined. These primary cells can be more useful than cultured cells because they can provide results closer to reality on functional analysis of bio-materials. When BALB/c mice splenocytes were incubated with different concentrations (0-125 g/mL) of ten LAB cell wall extracts for 24 h, inhibitory effect on mouse splenocyte proliferation was not observed at tested concentrations under the assay condition (Table 1). However, stimulatory effect on splenocyte proliferation was found for eight LAB cell wall extracts of *L. bulgaricus, L. paracasei, L. casei, L. acidophilus, L.* lactis*, S. thermophilus, B. breve,* and *P. pentosaceus* at the highest concentration of 125 g/mL tested. The other two LAB cell wall extracts of *L. plantarum* and *L. delbrueckii* did not greatly influence cell proliferation. Con A, the positive control, was only marginally effective (Table 1).

A similar inhibitory or stimulatory effect of LAB cell wall extracts on BALB/c mice mesenteric lymphocytes was observed (Table 1). Inhibition on mesenteric lymphocyte proliferation was not detected for the ten LAB cell wall extracts at all. The stimulatory effect on cell proliferation of BALB/c mice mesenteric lymphocytes was found for all LAB cell wall extracts except that of *L. delbrueckii*. On the other hand, PMA/ION positive control strongly stimulated the proliferation of mesenteric lymphocytes (Table 1).

Our data indicated that cell proliferation of mouse splenocytes and mesenteric lymphocytes could differ according to LAB strains and their concentrations used. Although all LAB cell wall extracts tested did not affect the proliferation of both immune cells of mouse, all LAB cell wall extracts except those of *L. plantarum* and *L. delbrueckii* had cell proliferative activity under the experimental condition (Table 1).

Several factors can influence lymphocyte proliferation induced by bacterial cell wall extract including LAB. Kitazawa *at al*. (1998) have shown that acidic extracellular polysaccharide precipitated from cell wall surface of *L. bulgaricus* 1073R-1 can stimulate the proliferative responses of murine splenocytes and Peyer's patches. The polysaccharide is a phosphopolysaccharide consisting of glucose, galactose, and phosphate. Kalka-Moll *et al*. (2002) also have shown that bacterial extracellular polysaccharides with zwitterionic charge spatial motif can elicite potent CD4 T-cell proliferative responses. Stingele *et al*. (2004) have indicated that the proliferative response might depend on the charge of free amino and carboxyl or phosphate groups within the repeated unit structure of the polysaccharide.

Such a mitogenic response of lymphocytes is not confined to extracellular polysaccharide from bacteria cell wall. Surface teichoic acid, lipoteichoic acid, and peptidoglycan consisting of bacteria cell wall are also known to stimulate the proliferative response of immune cells

Table 1. Effect of LAB cell wall extracts on cell proliferation of BALB/c mice splenocytes and mesenteric lymphocytes

Lactic acid bacteria	Conc. (g/mL)	Cell proliferation (%)	
		Splenocytes	Mesenteric lymphocytes
Con A	0	100.0±2.2[a]	-
	1	115.6±3.5[b]	
PMA / ION	0	-	100.0±1.7[a]
	50 / 7×10^{-7}		144.2±3.9[b]
Lactobacillus delbrueckii subsp. *bulgaricus*	0	100.0±2.2[a]	100.0±1.7[a]
	31	95.6±8.1[a]	99.6±2.4[a]
	62	100.0±1.6[a]	109.0±7.0[a]
	125	144.7±8.7[b]	144.1±11.2[b]
Lactobacillus paracasei	0	100.0±2.2[a]	100.0±1.7[a]
	31	101.9±2.2[a]	96.9±2.7[a]
	62	101.0±2.0[a]	105.4±0.4[a]
	125	121.0±3.7[b]	138.1±6.6[b]
Lactobacillus casei	0	100.0±2.2[a]	100.0±1.7[a]
	31	103.4±3.7[a]	126.6±2.4[b]
	62	104.3±5.5[a]	107.7±2.8[a]
	125	143.7±3.6[b]	136.6±8.1[b]
Lactobacillus acidophilus	0	100.0±2.2[a]	100.0±1.7[a]
	31	121.2±3.1[b]	106.1±1.5[a]
	62	126.2±3.3[b]	115.9±3.2[a]
	125	148.5±13.9[c]	160.4±1.4[b]
Lactobacillus plantarum	0	100.0±2.2[a]	100.0±1.7
	31	95.3±10.1[a]	98.2±6.5[a]
	62	91.3±6.5[a]	97.1±2.9[a]
	125	109.4±13.9[a]	115.6±4.7[b]
Lactobacillus delbrueckii	0	100.0±2.2[a]	100.0±1.7[a]
	31	94.4±1.1[a]	89.5±1.9[b]
	62	95.8±5.9[a]	115.3±1.9[c]
	125	100.7±4.6[a]	106.4±4.8[a]
Lactococcus lactis	0	100.0±2.2[a]	100.0±1.7[a]
	31	129.8±3.2[b]	139.3±6.2[b]
	62	131.9±10.0[b]	162.1±3.0[c]
	125	142.0±12.0[b]	171.0±9.1[c]
Streptococcus salivarius subsp. *thermophilus*	0	100.0±2.2[a]	100.0±1.7[a]
	31	137.5±1.5[b]	142.7±2.2[b]
	62	135.9±4.4[b]	146.4±9.0[bc]
	125	153.6±7.9[c]	156.8±4.4[c]
Bifidobacterium breve	0	100.0±2.2[a]	100.0±1.7[a]
	31	104.9±1.4[ab]	100.9±4.9[a]
	62	110.3±3.3[b]	107.6±7.3[a]
	125	119.1±4.8[c]	125.5±11.2[b]
Pedicoccus pentosaceus	0	100.0±2.2[a]	100.0±1.7[a]
	31	118.7±2.8[b]	113.3±2.8[bc]
	62	116.9±8.6[b]	112.0±5.4[b]
	125	120.2±4.2[c]	120.4±2.5[c]

(Laman *et al*., 2002; Räsänen and Arvilommi, 1981). LAB have Gram-positive cell walls mainly consisting of peptidoglycans, lipoteichoic acids, teichoic acids, proteins, and acidic extracellular polysaccharides (Delcour *et al*., 1999). Teichoic acids are polysaccharides of glycerol phosphate or ribitol phosphate linked via phosphodiester bonds (Brown *et al*., 2013). They have zwitterionic properties (Garimella *et al*., 2009). Therefore, the cell proliferative effect of LAB cell wall extracts on mouse splenocytes and mesenteric lymphocytes observed in this study might be due to these cell wall components. However, whether these cell wall components would induce mitogenic response of lymphocytes is unclear.

Effect of LAB cell wall extracts on T_H1 and T_H2 cytokine production

To determine the effect of LAB cell wall extract on T_H1 or T_H2 cytokine production, ten LAB cell wall extracts were assayed for their effect on the secretion of T_H1 cytokine (IFN-γ) and T_H2 cytokine (IL-4) of BALB/c mice splenocytes and mesenteric lymphocytes. When BALB/c mice splenocytes were treated with different concentrations (0-125 g/mL) of the ten LAB cell wall extracts, enhancement on IFN-γ production was observed for six LAB cell wall extracts of *L. bulgaricus, L. casei, L. acidophilus, L. plantarum, L. delbrueckii*, and *S. thermophiles*. The effect was relatively concentration-dependent (Table 2). Although the level of IFN-γ induced by these LAB cell wall extracts was much less than that of positive control Con A, the induced level was significant. For the other four cell wall extracts of *L. bulgaricus, L. casei, L. acidophilus*, and *S. thermophiles*, somewhat positive relation

Table 2. IFN-γ and IL-4 production of BALB/c mice splenocytes treated by LAB cell wall extracts

Lactic acid bacteria	Conc. (g/mL)	Cytokine (pg/mL)	
		IFN-γ	IL-4
Con A	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	1	416.0 ± 46.0[b]	106.9 ± 11.6[b]
Lactobacillus delbrueckii subsp. *bulgaricus*	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	88.0 ± 16.0[a]	26.4 ± 7.4[a]
	62	146.0 ± 11.0[b]	29.9 ± 5.9[a]
	125	194.5 ± 25.5[c]	31.1 ± 6.0[a]
Lactobacillus paracasei	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	46.0 ± 12.0[a]	24.5 ± 4.1[a]
	62	78.5 ± 15.5[a]	23.5 ± 8.2[a]
	125	102.0 ± 39.0[a]	29.9 ± 9.0[a]
Lactobacillus casei	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	93.5 ± 12.5[ab]	26.7 ± 7.7[a]
	62	129.5 ± 17.5[b]	24.0 ± 5.8[a]
	125	196.0 ± 24.0[c]	25.0 ± 8.1[a]
Lactobacillus acidophilus	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	84.5 ± 5.5[a]	26.9 ± 7.1[a]
	62	169.0 ± 13.0[b]	26.6 ± 7.9[a]
	125	230.0 ± 32.0[c]	28.2 ± 4.2[a]
Lactobacillus plantarum	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	100.0 ± 4.0[ab]	29.1 ± 5.7[a]
	62	123.5 ± 9.5[b]	27.5 ± 5.9[a]
	125	223.5 ± 18.5[c]	29.3 ± 5.3[a]
Lactobacillus delbrueckii	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	54.0 ± 38.0[a]	27.6 ± 5.4[a]
	62	129.5 ± 28.5[b]	27.4 ± 5.3[a]
	125	196.0 ± 17.1[b]	27.5 ± 6.0[a]
Lactococcus lactis	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	41.0 ± 3.0[b]	28.1 ± 7.1[a]
	62	66.5 ± 11.5[a]	30.6 ± 5.8[a]
	125	114.5 ± 21.5[a]	25.5 ± 8.3[a]
Streptococcus salivarius subsp. *thermophilus*	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	81.0 ± 21.0[a]	24.4 ± 9.2[a]
	62	129.0 ± 14.0[b]	26.5 ± 5.6[a]
	125	213.5 ± 10.5[c]	23.6 ± 7.6[a]
Bifidobacterium breve	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	36.5 ± 23.5[a]	28.9 ± 5.2[a]
	62	64.0 ± 8.0[a]	30.3 ± 6.6[a]
	125	85.5 ± 19.5[a]	28.5 ± 4.5[a]
Pedicoccus pentosaceus	0	74.0 ± 44.0[a]	26.0 ± 8.0[a]
	31	52.0 ± 5.0[a]	24.0 ± 6.1[a]
	62	84.0 ± 23.2[a]	26.1 ± 8.2[a]
	125	98.5 ± 19.3[a]	22.3 ± 4.6[a]

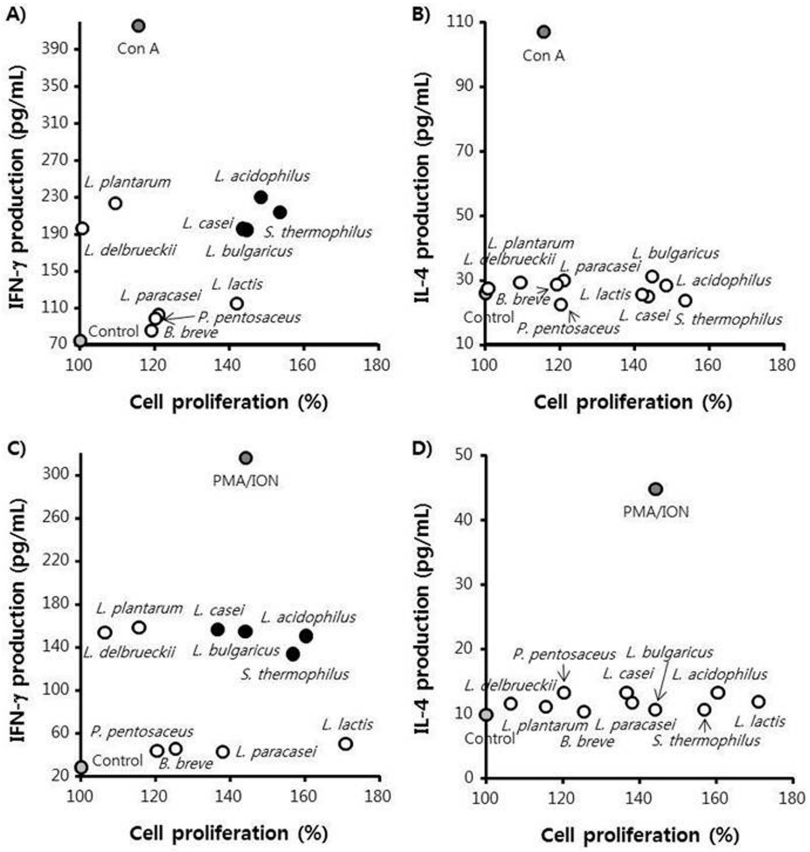

Fig. 1. Effect of LAB cell wall extracts on the relationship between cytokine production of IFN-γ or IL-4 and cell proliferation of BALB/c mice splenocytes and mesenteric lymphocytes. A) IFN-γ cytokine production versus cell proliferation in LAB cell wall extract-treated mice splenocytes; B) IL-4 cytokine production versus cell proliferation in LAB cell wall extract-treated mice splenocytes; C) IFN-γ cytokine production versus cell proliferation in LAB cell wall extract-treated mice mesenteric lymphocytes, D) IL-4 cytokine production versus cell proliferation in LAB cell wall extract-treated mice mesenteric lymphocytes.

was found between stimulating cell proliferation and enhancing IFN-γ production in splenocytes (Fig. 1A), suggesting that these LAB could promote cell proliferation and IFN-γ production at the same time. On the other hand, none of the ten LAB cell wall extracts increased IL-4 production of splenocytes. Only positive control Con A stimulated its secretion (Table 2, Fig. 1B).

The experiment was repeated with BALB/c mice mesenteric lymphocytes. As observed in splenocytes, increasing concentration of LAB cell wall extracts also increased IFN-γ production in mesenteric lymphocytes. The level of IFN-γ in mesenteric lymphocytes treated by the highest concentration of LAB cell wall extracts was higher than that without any treatment (Table 3). As in splenocytes, four LAB cell wall extracts of *L. bulgaricus, L. casei, L. acidophilus*, and *S. thermophilus* enhanced both cell proliferation and IFN-γ production (Fig. 1C) of mesenteric lymphocytes. However, LAB cell wall extract-medicated IL-4 production was not observed (Table 4, Fig. 1D). Meanwhile, PMA/ION positive control enhanced both IFN-γ and IL-4 productions in mesenteric lymphocytes (Table 4, Fig 1D). Lipopolysaccharide (LPS), a main component of outer membrane of Gram-negative bacteria, has been recognized as the main component in systemic inflammation and sepsis (Li *et al*., 2015). In this study, LAB used in the experiment of immunomodulatory activity are Gram-positive bacteria that are unable to release lipopolysaccharide. Thus, LAB treatment might have led to immune cell activation without inflammation. These results indicate that LAB used in this study have the potential to stimulate proliferation of both primary immune cells and IFN-γ production instead of IL-4 production.

Effect of LAB cell wall extracts on T_H1 and T_H2 polarization

Next, the influence of LAB cell wall extracts on T_H1 and T_H2 immune polarization of BALB/c mice spleno-

Table 3. IFN-γ and IL-4 production of BALB/c mice mesenteric lymphocytes treated by LAB cell wall extracts

Lactic acid bacteria	Conc. (g/mL)	Cytokine (pg/mL)	
		IFN-γ	IL-4
PMA / ION	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	50 / 7×10^{-7}	316.0 ± 54.1[b]	44.8 ± 3.6[b]
Lactobacillus delbrueckii subsp. *bulgaricus*	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	58.0 ± 14.1[b]	9.1 ± 3.8[a]
	62	86.2 ± 11.3[c]	9.0 ± 3.6[a]
	125	154.5 ± 14.5[d]	10.6 ± 3.8[a]
Lactobacillus paracasei	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	21.2 ± 13.2[a]	10.8 ± 5.0[a]
	62	33.5 ± 10.4[a]	9.9 ±5.4[a]
	125	42.1 ± 9.0[a]	11.7 ± 4.4[a]
Lactobacillus casei	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	58.6 ± 22.5[ab]	9.8 ± 2.7[a]
	62	69.3 ±17.5[b]	10.9 ± 2.8[a]
	125	156.2 ± 16.1[c]	13.2 ± 2.8[a]
Lactobacillus acidophilus	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	59.5 ± 9.5[b]	13.2 ± 2.0[a]
	62	84.1 ± 12.2[c]	12.8 ± 3.7[a]
	125	150.2 ± 12.0[d]	13.2 ± 3.5[a]
Lactobacillus plantarum	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	55.1 ± 9.0[a]	11.6 ± 4.6[a]
	62	83.4 ± 19.5[b]	11.6 ± 0.9[a]
	125	158.5 ± 16.5[c]	11.1 ± 0.7[a]
Lactobacillus delbrueckii	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	54 ± 38.0[ab]	13.5 ± 3.2[a]
	62	80.5 ± 38.5[b]	9.7 ± 3.0[a]
	125	153.2 ± 18.9[c]	11.6 ± 0.8[a]
Lactococcus lactis	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	26.1 ± 12.2[a]	14.5 ± 2.0[a]
	62	36.5 ± 11.4[ab]	13.6 ±4.0[a]
	125	49.7 ± 6.3[b]	11.8 ± 5.3[a]
Streptococcus salivarius subsp. *thermophilus*	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	56.3 ± 14.2[b]	10.8 ± 4.5[a]
	62	89.2 ± 16.0[c]	14.0 ± 2.4[a]
	125	133.5 ± 9.3[d]	10.6 ± 2.2[a]
Bifidobacterium breve	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	26.5 ± 13.3[a]	9.6 ± 2.8[a]
	62	44.1 ± 8.1[a]	9.8 ± 4.3[a]
	125	45.5 ± 10.6[a]	10.3 ± 4.6[a]
Pedicoccus pentosaceus	0	28.0 ± 12.0[a]	9.8 ± 3.2[a]
	31	17.0 ± 10.1[a]	10.4 ± 3.0[a]
	62	34.1 ± 3.3[a]	10.6 ± 5.4[a]
	125	43.5 ± 4.7[a]	13.2 ± 4.5[a]

cytes and mesenteric lymphocytes was determined. When mouse splenocytes were not treated by LAB cell wall extracts, the ratio of T_H1 cytokine (IFN-γ) to T_H2 cytokine (IL-4) was 3.4±2.7. The addition of Con A to the splenocytes slightly increased the ratio to 4.0±1.2. Such increase was not statistically significant. Therefore, Con A was considered to have no effect on T_H1 and T_H2 polarization of mice splenocytes. However, LAB cell wall extracts caused TH1 immune polarization of the splenocytes. Of the ten LAB cell wall extracts tested, six induced considerable level of T_H1 polarization of mice splenocytes. The best effect was observed for *S. thermophilus* (Table 4). The order of ability in inducing TH1 immune polarization of the six LAB cell wall extracts was: *S. thermophilus* (10.0) > *L. casei* (8.4) > *L. delbrueckii* (8.2) = *L. acidophilus* (8.2) > *L. plantarum* (8.0) > *L. bulgaricus* (6.3).

A similar tendency of the ten LAB cell wall extracts on T_H1 immune polarization of mice mesenteric lympho-

Table 4. Effect of LAB cell wall extracts on T_H1 and T_H2 polarization of BALB/c mice splenocytes and mesenteric lymphocytes

Lactic acid bacteria	Conc. (g/mL)	Ratio of IFN-γ / IL-4	
		Splenocytes	Mesenteric lymphocytes
Con A	0	3.4 ± 2.7[a]	-
	1	4.0 ± 1.2[a]	
PMA/ ION	0	-	3.6 ± 3.4[a]
	50 / 7×10^{-7}		7.0 ± 0.9[b]
Lactobacillus delbrueckii subsp. *bulgaricus*	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	3.4 ± 0.5[a]	6.9 ± 1.8[ab]
	62	5.2 ± 2.0[ab]	12.1 ± 8.7[bc]
	125	6.3 ± 0.6[b]	16.1 ± 6.1[c]
Lactobacillus paracasei	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	1.9 ± 0.3[a]	3.2 ± 3.7[a]
	62	3.5 ± 0.8[a]	4.0 ± 1.6[a]
	125	3.3 ± 0.4[a]	4.5 ± 3.5[a]
Lactobacillus casei	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	4.0 ± 2.3[a]	7.1 ± 5.9[a]
	62	5.5 ± 0.9[ab]	6.4 ± 0.1[a]
	125	8.4 ± 2.5[b]	12.7 ± 5.5[b]
Lactobacillus acidophilus	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	3.4 ± 1.6[a]	4.5 ± 2.0[a]
	62	7.1 ± 3.7[b]	6.9 ± 1.5[a]
	125	8.2 ± 0.1[b]	12.0 ± 3.2[b]
Lactobacillus plantarum	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	3.6 ± 1.2[a]	6.0 ± 4.4[a]
	62	4.8 ± 1.9[a]	7.4 ± 3.2[a]
	125	8.0 ± 2.9[b]	14.5 ± 3.4[b]
Lactobacillus delbrueckii	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	2.3 ± 2.6[a]	5.0 ± 5.7[a]
	62	3.3 ± 2.4[a]	8.2 ± 0.6[a]
	125	8.2 ± 1.7[b]	13.3 ± 3.5[b]
Lactococcus lactis	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	1.5 ± 0.4[a]	1.9 ± 1.5[a]
	62	2.3 ± 1.2[a]	3.2 ± 2.5[a]
	125	5.3 ± 3.6[a]	5.6 ± 4.3[a]
Streptococcus salivarius subsp. *thermophilus*	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	4.2 ± 3.5[a]	5.6 ± 1.5[a]
	62	5.0 ± 0.7[a]	6.8 ± 3.3[a]
	125	10.0 ± 3.9[b]	13.3 ± 5.1[b]
Bifidobacterium breve	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	1.2 ± 0.9[a]	2.6 ± 0.9[a]
	62	2.2 ± 0.3[a]	6.0 ± 4.9[a]
	125	3.2 ± 1.7[a]	5.0 ± 1.7[a]
Pedicoccus pentosaceus	0	3.4 ± 2.7[a]	3.6 ± 3.4[a]
	31	2.3 ± 0.5[a]	2.1 ± 2.2[a]
	62	3.9 ± 3.0[a]	3.5 ± 1.7[a]
	125	4.8 ± 2.6[a]	3.9 ± 2.3[a]

cytes was observed. The ratio of T_H1 cytokine to T_H2 cytokine in the control without any treatment was 3.6±3.4. Of the ten LAB cells extracts, six extracts (*L. bulgaricus, L. plantarum, S. thermophilus, L. delbrueckii, L. casei*, and *L. acidophilus*) significantly induced T_H1 immune polarization. Their effect was better than that of positive control PMA/ION mixture (Table 4). However, unlike that in splenocytes, the best effect in mesenteric lymphocytes was observed for *L. bulgaricus* cell wall extract. The order of ability in inducing TH1 immune polarization of the six LAB cell wall extracts was: *L. bulgaricus* (16.1) > *L. plantarum* (14.5) > *S. thermophilus* (13.3) = *L. delbrueckii* (13.3) > *L. casei* (12.7) > *L. acidophilus* (12.0).

Our results showed that LAB cell wall extracts pro-

moted T_H1 cytokine (IFN-γ) production instead of T_H2 cytokine (IL-4) production in BALB/c mice splenocytes and mesenteric lymphocytes. Although the extent of T_H1 cytokine IFN-γ production induced by tested LAB cell wall extracts was differed according to strains (Table 2, Table 3 and Fig. 1), our results were very similar to results of many studies reporting that LAB increased T_H1 cytokines such as IL-2, IL-12, IFN-γ, and tumor necrosis factor (TNF)- but reduced T_H2 cytokines such as IL-4, IL-5, IL-10, and IL-13 in various immune cells (Fujiwara *et al*., 2004; Ghadimi *et al*., 2010; Iwabuchi *et al*., 2007; Kimoto *et al*., 2004; Pochard *et al*., 2002). These T_H1 and T_H2 cytokines carry out important functions in host immune system. T_H1 cytokines mediate cell-mediated immunity, including attacking intracellular pathogens such as viruses, regulating classic delayed-type hypersensitivity skin response to viral and bacterial antigens, and eliminating cancer cells. However, T_H2 cytokines mostly induce humoral immunity related to allergy and immunoglobulin (Ig) E-based disease (Ghadimi *et al*., 2008; Kidd, 2003). Of these T_H1 and T_H2 cytokines, INF-γ, one of T_H1 cytokines and a major macrophage activating lymphokine, has been known to suppress the induction of these T_H2 cytokines (Behera *et al*., 2002; Holt *et al*., 1997; van der Velden *et al*., 2001).

As shown in Table 4, the ability of LAB cell wall extracts to polarize T_H1 immune response via stimulating IFN-γ production may be important for homeostasis maintenance in the host since various diseases have been associated with imbalanced T_H1 and T_H2 immune response. In particular, several studies have shown that food allergy and atopic disease are closely associated with the overproduction of IgE antibody induced by T_H2 immune response (Palomares, 2013; Rautava *et al*., 2004). LAB can alleviate allergic disorders via modulating host T_H1/ T_H2 immune response (Khani *et al*., 2012; Özdemir, 2010). Such LAB-mediated effect is partly due to immune-active components such as peptidoglycan, one of their cell wall surface constituents (Amrouche *et al*., 2006).

Previously, we have investigated the surviving ability of the ten commercial LAB against the exposure to ionizing radiation. Of the ten LAB tested, *L. acidophilus* was the most radio-resistant LAB (Hwang *et al*., 2013). In this study, the ability of the ten LAB in inducing cell proliferation of immune cells and production of cytokines such as IFN-γ and IL-4 was evaluated. As a result, four LAB (*L. acidophilus*, *L. bulgaricus*, *L. casei*, and *S. thermophilus*) were found to have mitogenic and T_H1 immune polarizing abilities. These results could be useful for selecting probiotics for maintaining astronauts' immune system during their space missions.

In conclusion, ten LAB used in the production of ferment milk products were tested for their capability to stimulate mitogenic and T_H1 immune responses. Of the ten LAB, four (*L. acidophilus*, *L. bulgaricus*, *L. casei*, and *S. thermophiles*) were able to induce cell proliferating response and simulate polarized TH1 immune reaction by stimulating the production of T_H1 cytokine IFN-γ without influencing the production of T_H2 cytokine IL-4 in mouse immune cells. Since LAB are indigenous inhabitants of healthy intestine, they have been safely used in the production of fermented foods from ancient times. Our results suggest that LAB tested in this study might be possible probiotics candidates as direct-fed microbes for astronauts under extreme space environments and for general people under normal conditions. Further studies such as comparing cell wall components, acid and bile intolerance, and potent intestine adhesive ability of these LAB are required to select the best LAB strains suitable as specialized purposed foods for extreme environment such as space, desert, and deep sea.

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