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# Isolation and Characterization of an Immunopotentiating Factor from *Lactobacillus plantarum* in *Kimchi*: Assessment of Immunostimulatory Activities

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**Abstract** The immunostimulatory activities of *Lactobacillus plantarum*, the major microorganism in *kimchi* fermentations were investigated. Five strains of *L. plantarum* exhibited weak immunopotentiating activity, but *L. plantarum* PS-21 showed as strong a mitogenic activity as *Bifidobacterium adolescentis* M101-4, a known positive strain. It is of interest that, *L. plantarum* PS-21 stimulated proliferation of Peyer's patch cells, one of the most important tissues in the gut-associated lymphoreticular system. Cell wall fractions from *L. plantarum* PS-21 also showed strong mitogenic activity compared with the soluble cytoplasmic fraction. A peptidoglycan fraction (PG) extracted from the cell wall of *L. plantarum* PS-21 was identified as an active mitogenic component when used in murine lymph node and spleen cell test systems. PG showed dose-dependent mitogenic activity and significantly enhanced antibody production in lymph node cells when studied *in vitro*. The lysosomal enzyme activity of murine peritoneal macrophages was increased when analyzed following injection of PG to the host animal. Furthermore, PG enhanced the production of cytokines such (TNF-α and IL-6) in the *in vitro* culture of RAW 264.7 macrophage cells.

**Keywords:** *Lactobacillus plantarum*, peptidoglycan, immunostimulating activities

## Introduction

It has been reported that lactic acid bacteria provide beneficial effects in the prevention or treatment of intestinal disorders and in enhancing host immune responses. Thus, Vitini *et al.* (1) and Perdigon *et al.* (2) have described a gut mucosal immunostimulatory property for lactic acid bacteria. An immunoadjuvant activity of *Lactobacillus casei* (3), considerable antitumor activity of *L. casei* (4), the potentiation of immunoglobulin A responses (5), and the prevention of microbial infections (6) have also been reported. Mitsuoka (7) demonstrated that Bifidobacterium, the predominant intestinal flora microorganism, play an important role in the physiological and immunological system of the host. Further, a number of investigations have suggested that the bifidobacteria can be characterized by a variety of immune functions including mitogenicity (8), adjuvant activity (9), macrophage activation (8), antitumor activity (10), and antibody production (11, 12). In the previous study, several foods were screened for their microorganism content and any mitogenic activity and antibody production. *Bifidobacterium adolescentis* M101-4 demonstrated a strong immunopotentiating activity and was selected as a standard strain for further studies (13). In addition, the immunostimulative activities of commercial bifidobacteria have been investigated (14). Although the above-mentioned activities were demonstrated, the molecular and cellular mechanisms were not clarified. To characterize immunostimulant properties of bacteria, it is essential to identify their active component(s) since the bacteria exhibit various factors which may act as activators or suppressors.

*Kimchi* has long been consumed as a traditional food fermented by endogenous lactic acid bacteria. Over 180 types of *kimchi* are currently classified according to their major ingredients. Recently, the scientific importance of *kimchi*, especially in Korea, has increased because the food is considered as nutritionally valuable and is also believed to have antioxidative, antimutagenic, and anticancer properties (15). However, little is known about the immunopotentiating properties of the lactic acid bacteria normally found in *kimchi*. The major microorganism in *kimchi* fermentation, *Lactobacillus plantarum*, gives the proper acidity and a unique taste that affects *kimchi* quality (16). *L. plantarum*, is present in large quantities following initial *kimchi* fermentation and produces maximum acidity at a later stage (17).

In the present study, *L. plantarum* strains with high immunopotentiating properties were selected by screening for mitogenic activity using cells from the murine lymph node, spleen and Peyer's patches. To characterize possible immunopotentiators of selected bacteria, intact cells, fragmented cell walls, soluble cytoplasmic fractions, and the peptidoglycans fraction of the bacteria were prepared. Lymphocyte proliferation, antibody production, macrophage activation, and cytokine production were then investigated in the test cell systems. Our objective was to develop a physiologically functional food capable of maintaining or positively stimulating the host defense system that is otherwise lost as a consequence of increasing age.

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## Materials and Methods

**Microorganisms** Pure strains of *B. adolescentis* M 101-4 were purchased from the Japan Bifidus Foundation (Tokyo, Japan). *L. plantarum* KCTC 1048, 3099, and 3107 were obtained from the Korean Collection for Type Cultures (Daejeon, Korea) while the *L. plantarum* PS-21, JK-08, and HS-15 strains were isolated from commercial factory-manufactured *kimchi* using Lactobacillus selective (LBS) agar media with slight modifications (18, 19). The isolated lactic acid bacteria were identified using the Microstation 5.0 bacteria and yeast identification system (Biolog Inc., Hayward, CA, USA). All bacterial strains were cultured and subcultured using MRS broth (Difco Laboratories, Detroit, MI, USA) kept at 37°C until the final log phase was reached. After the fermentation, the cells were harvested using a refrigerated centrifuge, washed three times with distilled water and lyophilized for assay. For the long term storage of each strain, aseptically harvested bacterial cells were resuspended in 10%(w/v) non fat dry milk (NDM; Difco) containing 10%(v/v) glycerol (Sigma Chemical Co., St. Louis, MO, USA) and stored at -80°C.

**Mice** Eight-week old male BALB/c mice were obtained from Daehan Biolink Co., Ltd. (Umsong, Chungbuk, Korea). The mice were acclimated for one week in a pathogen-free animal facility and then sacrificed by cervical dislocation while under light ether anesthesia before tissue and cell isolation and their use in the experiments.

**Proliferation assay of lymph node cells** The mice were immunized subcutaneously at the base of the tail and in a hind foot pad with 50 μg of bovine serum albumin (BSA; Sigma) emulsified in phosphate-buffered saline (PBS) and complete Freund's adjuvant (Bacto-adjuvant complete H37Ra; Difco). After two weeks, draining lymph node cells from these mice were dispersed to provide a single-cell suspension and plated on 96-well tissue culture plates (Costar, Cambridge, MA, USA) at $5\times10^4$ cells per well (200 μL/well) in RPMI 1640 medium containing 10%(v/v) fetal bovine serum (Gibco Laboratories, Chagrin Falls, OH, USA), 50 μg 2-mercapto-ethanol, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Various doses of each microorganism (in triplicate) were added to the wells and received the Concanavalin A mitogen (Con A, Type IV; Sigma) and lipopolysaccharides (LPS) from *Salmonella typhimurium* (Sigma) or the homologous antigen, and the lymph node cells incubated at 37°C in 5% $CO_2$ for 72 hr. Proliferation was detected using a colorimetric assay (20) with slight modifications. Briefly, all the cultures were added with 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) solution (5 mg in 1 mL PBS) and after a further 6 hr incubation, 100 μL of isopropanol with 0.04 N HCl was added to each well. Within 1 hr, the absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm on a microplate spectrophotometer (Spectra Max 340PC; Molecular Devices, Sunnyvale, CA, USA).

**Proliferation assay of spleen and Peyer's patch cells** Spleen cells were prepared aseptically. Peyer's patch cells were obtained according to the method of Suzuki *et al.* (21). Briefly, the small intestine was aseptically exposed (sterile paper) and the Peyer's patches carefully dissected and teased gently using two glass slides and RPMI 1640 medium. Single cell suspensions from the spleen and Peyer's patch were cultured to obtain a final density of $5\times10^4$ cells/mL in the 96-well tissue culture plates. Triplicate wells were challenged with various doses of each sample, and then the cells were incubated at 37°C in 5% $CO_2$ for 48 hr. Cell proliferation was detected by an MTT assay as mentioned above.

**Preparation of cell fractions from *L. plantarum* PS-21** *L. plantarum* PS-21 was cultured in MRS broth (Difco) for two days at 37°C. The cells were then harvested and washed three times with distilled water. The harvested whole cells were suspended in distilled water and fragmented twice using a cell disrupter (Branson, Danbury, CT, USA) for 15 min under ice-cold conditions. The homogenate was then centrifuged twice (1,000×g for 20 min each) until the intact cells were fragmented and the disrupted product then centrifuged (70,000×g for 30 min at 5°C). The resulting precipitate and supernatant were lyophilized and designated as cell wall and cytoplasmic fraction, respectively. The cell wall fraction was treated with mutanolysin (Sigma) in 50 μM phosphate buffer containing 4 μM $MgCl_2$ at 37°C for 14 hr, and then fractionated by a membrane with a molecular weight cut-off value of 50,000 (Millipore, Bedford, MA, USA). The high molecular weight fraction was used as a mutanolysin digest. The isolation of peptidoglycans (PG) from the cell wall fractions was then performed according to the method of Sekine *et al.* (22).

***In vitro* antibody production assay** After a 2 week immunization period with 50 μg of BSA, draining lymph node cells were plated in 48-well tissue culture plates at $2\times10^6$ cells per well (1 mL/well). Triplicate wells were challenged with various doses of each sample, and the cells incubated at 37°C in 5% $CO_2$. As positive controls, 10 μg/mL of LPS and 50 μg/mL of BSA were also tested. The upper 0.5 mL of the culture was then discarded, and 0.5 mL of fresh RPMI 1640 medium was added at the 3rd and 7th day of the culture period. After a further 4 days, the plates were centrifuged, and the culture supernatants collected. The anti-BSA antibody titer in each supernatant was measured by an enzyme-linked immunosorbant assay (ELISA) at 405 nm (23).

**Enzyme activity of PG-induced peritoneal macrophages** Peritoneal macrophages were collected 2 days after an intraperitoneal injection of 2 mL of 3% Bacto-Peptone (Difco) or 100 μg of PG. Resident peritoneal macrophages were also collected as a control. The induced macrophages ($2\times10^5$ cells/200 μL/well) were cultured at 37°C for 24 hr. The activity of β-glucuronidase and β-galactosidase in the culture supernatants was determined by the method of Stossel (24) and Conchie *et al.* (25) using the synthetic substrates p-nitrophenyl-β-D-glucuronide and o-nitrophenyl-β-D-galactopyranoside, respectively.

**Culture conditions for the RAW 264.7 macrophage cell line** The RAW 264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle medium supplemented with 1%(v/v) NTCT 135, and RPMI-1640 medium supplemented with 2-mercaptoethanol (50 μM) and non-essential amino acids (1 mM). Both media were further supplemented with 10%(v/v) fetal bovine serum (Gibco), 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. RAW 264.7 cells were cultured to obtain a final density of $5\times10^5$ cells/mL in 48-well flat-bottomed tissue culture plates (Costar) with and without 1 μg/mL of LPS. Triplicate cultures were exposed to various concentrations of samples and were incubated for two days at 37°C in 5% $CO_2$. After incubation, the supernatants were separated and stored at -20°C for cytokine assay.

**Determination of cytokine production** Cytokine production in culture supernatants was assessed with an ELISA by using modifications of the procedure described by Dong *et al.* (26). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μL/well of 1 μg/mL purified IL-6 or TNF-α antibody (rat anti-mouse; PharMingen, San Diego, CA, USA) diluted in 0.1 M sodium bicarbonate buffer (pH 8.2). Plates were washed three times with 0.01 M PBS (pH 7.2) containing 0.2%(v/v) Tween 20 (PBST) to remove excess capture antibody. To reduce non-specific binding, the wells were blocked with 300 μL of PBST containing 3%(w/v) BSA for 30 min at 37°C and washed four times with PBST. Standard recombinant IL-6, TNF-α, or samples, diluted in PBS, were added in 50 μL aliquots to appropriate wells. Plates were incubated for 1 hr at 37°C. After washing 4 times with PBST, biotinylated rat anti-mouse IL-6 or TNF-α antibody was diluted in PBST-BSA to 2 μg/mL and 50 μL was added to each well. The plates were incubated for 1 hr at room temperature. The plates were washed 6 times with PBST and incubated with 50 μL/well of streptavidin-horseradish peroxidase conjugate (1.5 μg/mL in PBST-BSA; Sigma) at room temperature for 1 hr. After washing eight times, bound peroxidase conjugate was detected by adding 100 μL/well of substrate solution consisting of 0.1 M citric-phosphate buffer (pH 5.5), 0.4 mM of tetramethylbenzidine (Sigma) and 1.2 mM of 1%(v/v) $H_2O_2$. An equal volume of stopping reagent consisting of 6 N $H_2SO_4$ was added to each well to stop the reaction and absorbance was measured at 450 nm on a microplate spectrophotometer (Spectra Max 340PC; Molecular Devices). The cytokines were quantified by using a standard curve and the Softmax curve-fitting program (Molecular Devices).

## Results and Discussion

**Proliferation of BSA-primed lymph node cells** The growth stimulation properties of *L. plantarum* towards lymph node cells primed with BSA was assessed for an immunopotentiating activity. In this assay, mitogenicity to lymph node cells was completely induced by Con A and LPS. In our previous study (13), we had screened food related microorganisms for their immunopotentiating activity and selected *B. adolescentis* M101-4 which demonstrated strong mitogenic properties and enhanced antibody production. As shown in Table 1, *B. adolescentis* M101-4 displayed a strong immunopotentiating activity towards the proliferation of the murine lymph node cells. Five different strains of *L. plantarum* exhibited weak immunopotentiating activity compared to *B. adolescentis* M101-4, but of the five, *L. plantarum* PS-21 showed the strongest mitogenic activity, comparable to *B. adolescentis* M101-4. These results, therefore, indicate that the immunopotentiating activity of the food related microorganisms was dependent on the properties of each individual strain. Thus, further study could involve assessment of any actual physiological functions specific for a particular *kimchi.*

**Proliferation of spleen and Peyer's patch cells** The proliferative response of unprimed spleen cells were tested to assess the mitogenic activities of bifidobacteria. As shown in Table 2, *B. adolescentis* M101-4 and *L. plantarum* PS-21 showed strong mitogenic activity, whereas *L. plantarum* KCTC 1048 and *L. plantarum* KCTC 3099 demonstrated remarkably activities. Interestingly, *L. plantarum* PS-21 expressed especially strong mitogenic activity toward the Peyer's patch cells, a cell population considered to be one of the most important in gut-associated lymphoreticular tissues, and to play an important role in gastrointestinal immune responses (27, 28). Therefore, it is expected that *L. plantarum* PS-21 could be a source that endows a food with important health promoting properties by augmenting host defenses against an infectious foreign toxic microorganisms and aberrant tumor cells.

**Mitogenic activity of cell fractions from *L. plantarum* PS-21** The proliferative response of BSA-primed lymph node and spleen cells was tested in an attempt to determine mitogenic activity for the cell fractions from *L. plantarum* PS-21. A protein-free endotoxin, LPS, was used as a positive control in this study. As shown in Table

**Table 1. Proliferative response of murine lymph node cells**

| Treatments | Dose (μg/mL) | Proliferation[1)] ($O.D._{570-630}$) |
|---|---|---|
| Control | - | 0.22±0.15[2)] |
| LPS | 10 | 1.21±0.30 |
| Con A | 4 | 1.05±0.44 |
| BSA | 50 | 0.78±0.13 |
| *B. adolescentis* M101-4 | 50 | 0.84±0.22 |
| *L. plantarum* KCTC 1048 | 50 | 0.46±0.27 |
| *L. plantarum* KCTC 3099 | 50 | 0.37±0.14 |
| *L. plantarum* KCTC 3107 | 50 | 0.32±0.29 |
| *L. plantarum* PS-21 | 50 | 0.83±0.16 |
| *L. plantarum* JK-08 | 50 | 0.50±0.32 |
| *L. plantarum* HS-15 | 50 | 0.38±0.19 |

[1)]BSA-primed lymph node cells ($5\times10^4$ cells/well) were cultured at 37 °C in 5% $CO_2$ for 72 hr with various bacterial samples.
[2)]Results are expressed as the mean's from three independent experiments.

**Table 2. Proliferative response of murine spleen and Peyer's patch cells**

| Treatments | Dose (μg/mL) | Proliferation[1] ($O.D._{570-630}$) | |
|---|---|---|---|
| | | Spleen | Peyer's patch |
| Control | - | 0.28±0.11[2] | 0.19±0.08 |
| LPS | 10 | 1.30±0.21 | 1.14±0.16 |
| Con A | 4 | 1.03±0.36 | 0.98±0.24 |
| *B. adolescentis* M101-4 | 50 | 0.94±0.07 | 0.72±0.26 |
| *L. plantarum* KCTC 1048 | 50 | 0.24±0.18 | 0.31±0.06 |
| *L. plantarum* KCTC 3099 | 50 | 0.31±0.08 | 0.28±0.13 |
| *L. plantarum* KCTC 3107 | 50 | 0.41±0.24 | 0.43±0.12 |
| *L. plantarum* PS-21 | 50 | 0.78±0.10 | 0.88±0.30 |
| *L. plantarum* JK-08 | 50 | 0.68±0.35 | 0.66±0.21 |
| *L. plantarum* HS-15 | 50 | 0.47±0.12 | 0.38±0.23 |

[1]Spleen and Peyer's patch cells ($5\times10^4$ cells/well) were cultured at 37 °C in 5% $CO_2$ for 48 hr with various bacterial samples.
[2]Results are expressed as the mean's from three independent experiments.

3, the cell wall fraction from *L. plantarum* PS-21 had a strong mitogenic activity when compared with the soluble cytoplasmic fraction, but the activity was markedly decreased by treatment with mutanolysin, an agent that hydrolyzes glycoside linkages between the N-acetylmuramic acid and N-acetylglucosamine in the cell wall of Gram-positive bacteria (29). Also, the mitogenic activity of PG fraction was shown to be greater than that of cell wall fraction. These results suggest that PG is the active component in the cell wall for the observed mitogenic effects. To identify a possible immune property of bacteria, it was necessary to first purify and then characterize active components because of the content of diverse and variable active and inhibitory components that act through different mechanisms serving to complicate any interpretation of forthcoming results. In this study, we purified the active PGs fraction from *L. plantarum* PS-21, which was seen to enhance lymphocyte proliferation and antibody production. While an immunologic property of the PGs fraction from *L. plantarum* PS-21 has not as yet been reported, it would not be surprising since the PGs are the main constituents of the cell wall in most Gram-positive bacteria. Thus, a variety of biological activities including mitogenicity, adjuvanticity, and antitumor activity have been reported for these compounds (9, 10, 22, 30, 31). Numerous studies have been performed regarding the fractions and/or components of bacteria shown to possess immunological properties. Kitazawa *et al.* (32) reported that a slime product from *Lactococcus lactis* ssp. *cremoris* had B cell mitogenic activity and induced macrophage activation. Takahashi *et al.* (11) reported that mice fed bacterial fractions, developed an antibody response that was enhanced with cytoplasm of *B. longum* and both the cytoplasm and cell wall fractions of *L. acidophilus*. Most reports suggest a potential immunologic property of whole cells or cell wall fractions of the bacteria. We assume that the difference in location of active components in bacteria, therefore, depends upon the fractionation methods, assays, animal models, and more likely, the specificities of each bacterial strain that is tested.

**Table 3. Specific mitogenic activities of peptidoglycans from *L. plantarum* PS-21 on the proliferative response of murine lymph node and spleen cells**

| Treatments[1] | Dose (μg/mL) | Proliferation[2] ($O.D._{570-630}$) | |
|---|---|---|---|
| | | Lymph node | Spleen |
| Control | - | 0.18±0.07[3] | 0.21±0.11 |
| LPS | 10 | 1.12±0.21 | 1.31±0.27 |
| Whole cell | 50 | 0.62±0.18 | 0.72±0.09 |
| Sonicated cell | 50 | 0.72±0.24 | 0.78±0.13 |
| Cytoplasmic fraction | 50 | 0.45±0.33 | 0.48±0.15 |
| Cell wall fraction | 50 | 0.81±0.22 | 0.83±0.03 |
| Mutanolysin digest | 50 | 0.33±0.19 | 0.28±0.08 |
| Peptidoglycans | 10 | 0.79±0.16 | 0.88±0.18 |
| | 50 | 0.93±0.24 | 0.96±0.14 |
| | 100 | 1.08±0.09 | 1.18±0.12 |

[1]The harvested *L. plantarum* PS-21 (whole cells) were broken with a cell disrupter water (sonicated cell), and then ultracentrifuged resulting in precipitate (cell wall) and supernatant (cytoplasmic) fractions.
[2]Lymph node cells and spleen cells ($5\times10^4$ cells/well) were cultured at 37°C in 5% $CO_2$ with various bacterial samples.
[3]Results are expressed as the means from three independent experiments.

**Enhancement of antibody production** The lymph node cells derived from BSA-immunized BALB/c mice were cultured with and without each sample, and the anti-BSA antibody titer of the culture supernatant was tested. When the homologous antigen was added, BSA-dependent antibody production was observed (Fig. 1). LPS, the B cell mitogen, also augmented antibody production by BSA-primed lymph node cells. Whole cell and cell wall fractions enhanced the antibody titer, while the soluble cytoplasmic fraction was shown to stimulate a low level of antibody production. On the other hand, PG significantly enhanced the anti-BSA antibody titer and demonstrated a near doubling of the soluble cytoplasmic fraction when tested at the same dose. These results suggested that PG from *L. plantarum* PS-21 stimulated B cells directly and resulted in a proliferation of B cells and antibody production (as per B cell mitogen). Most of the B cell mitogens induce cell division, DNA synthesis, and blast transformation in a high percentage of B lymphocytes. Unlike immunogens which activate only lymphocytes that bear specific receptors, B cell mitogens activate many B lymphocytes in a nonspecific manner irrelevant of the antigenic specificity of the immunoglobulin receptor (33). Because of this feature, B cell mitogens are able to induce differentiation of B lymphocytes into antibody-producing cells, resulting in the synthesis and secretion of soluble polyclonal antibodies. We also have determined the IgG isotypes for which secretion was enhanced in the presence of the cell wall and PG (Fig. 1). Both cell wall and PG preparations induced strong upregulation of IgG secretion compared to that of IgM, although the IgM level was higher than the total IgG titer of the control culture. It has

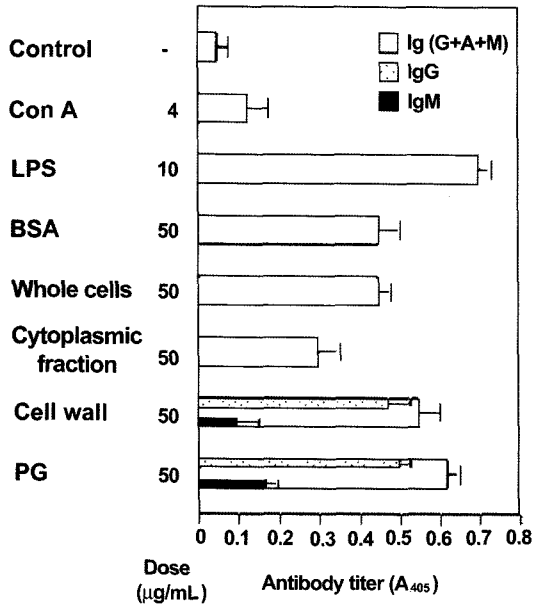


**Fig. 1. *In vitro* antibody production of BSA-primed lymph node cells.** BSA-primed lymph node cells ($2\times10^6$ cells/mL) were cultured at 37°C in 5% $CO_2$ for 11 days with various bacterial samples and the antibody titer of culture supernatants was measured by an ELISA at 405 nm. Results are expressed as the mean's for triplicate experiments.

been reported that lactic acid bacteria and bifidobacteria enhance the phagocytic activities of murine macrophages (34, 35) and blood cells from the human (36), however, the molecular and cellular mechanisms responsible have not been clarified. It is possible that following the stimulation of the B lymphocytes by the bacteria and bacterial fractions, the upregulated secreted antibodies partially enhance phagocytic properties of the granulocytes, monocytes, and macrophages. We speculate this to be possible because the phagocytes can recognize a variety of pathogens and tumor cells by binding serum factors, for example, IgG as an opsonin, and IgM as an activator.

**Augmentation of enzyme activities** Perdigon *et al.* (35) have reported that *L. casei* and *L. bulgaricus* stimulated lymphocytes by inducing the release of lysosomal enzymes from peritoneal macrophages and by activating cells of the phagocytic mononuclear system. Even though the enzyme release process can not always be directly related to phagocytosis, *in vivo* activation of macrophages is important in immunopotentiation and the suppression of tumor growth. As shown in Table 4, the enzyme activity of peritoneal macrophages was increased slightly by peptone injection, and more significantly by PG-induction. These results indicate that PG enhances macrophage activity *in vivo*.

**Effect of PG on cytokine production** Cytokine production and release are important functions of activated macrophages, and two cytokines in particular, TNF-α and IL-6, were identified in the culture supernatant from the RAW 264.7 macrophages with/without LPS (Table 5, 6). LPS, one of the most powerful activators of macrophages

**Table 4. Effect of peptidoglycans from *L. plantarum* PS-21 on enzyme activities of murine peritoneal macrophages *in vivo***

| Macrophages[1)] | Glucuronidase (nmol PNP[2)]/hr) | Galactosidase (nmol ONP[2)]/hr) |
|---|---|---|
| **Experiment I** | | |
| Resident | 3.2±1.3[3)] | 2.8±0.7 |
| Peptone-induced | 7.8±2.4 | 10.1±1.6 |
| PG-induced | 21.3±3.1 | 18.7±2.2 |
| **Experiment II** | | |
| Resident | 2.6±1.1 | 2.9±1.5 |
| Peptone-induced | 8.8±2.0 | 13.4±2.2 |
| PG-induced | 19.2±1.8 | 18.3±0.9 |

[1)]Peritoneal macrophages were collected two days after an intraperitoneal injection of 2 mL of 3% Bacto-Peptone (peptone-induced) or 100 μg of PG (PG-induced). Resident peritoneal macrophages (none injected) were also collected as a control.
[2)]Abbreviation: PNP, p-nitrophenyl-β-D-glucuronide; ONP, o-nitrophenyl-β-D-galactopyranoside.
[3)]Results are expressed as the means for triplicate experiments.

augmented production of all cytokines, whereas the fold increase when cultured with LPS-stimulated cells was less. PG significantly stimulated the RAW 264.7 macrophages to produce cytokines. In general, the macrophage-derived cytokines of TNF-α and IL-6 have been shown to play a role in the activation of the immune system, the induction of an acquired immunity against pathogenic infections and the transduction of apoptotic signals in tumor cells (37). There is an extensive body of evidence to demonstrate that cytokines play roles in host defenses, inflammatory responses, and autoimmune disease (37). It has also been reported that lactic acid bacteria and other non-pathogenic bacteria induce cytokine production (38). Further, Sekine *et al.* (10) proposed a critical role of cytokines to the antitumor properties of *B. infantis*. In conclusion, PG was demonstrated to enhance the proliferation of murine lymphocytes, antibody production, and macrophage activation both *in vivo* and *in vitro*. It should prove interesting going forward to explore possible relationships between the

**Table 5. Effect of peptidoglycans from *L. plantarum* PS-21 on *in vitro* TNF-α production by unstimulated (LPS-) and LPS-stimulated (LPS+) RAW 264.7 cells**

| Treatments | Dose (μg/mL) | TNF-α relative change[1)] (fold control) | |
|---|---|---|---|
| | | LPS- | LPS+ |
| Whole cell | 100 | 6.2±0.8[2)] | 2.7±1.0 |
| Cell wall | 100 | 7.3±1.3 | 3.3±0.8 |
| Peptidoglycans | 10 | 7.6±1.7 | 6.5±0.9 |
| | 50 | 22.3±1.0 | 8.4±1.6 |
| | 100 | 31.1±1.4 | 8.3±0.3 |

[1)]RAW 264.7 cells ($5\times10^5$ cells/mL) were cultured at 37°C in 5% $CO_2$ with various samples under unstimulated (LPS-) or 1 μg/mL of LPS-stimulated (LPS+) conditions, and TNF-α production in the culture supernatants was assessed by ELISA.
[2)]Results are expressed as the mean's of fold increase for triplicate experiments.

**Table 6. Effect of peptidoglycans from *L. plantarum* PS-21 on *in vitro* IL-6 production by unstimulated (LPS-) and LPS-stimulated (LPS+) RAW 264.7 cells**

| Treatments | Dose (μg/mL) | IL-6 Relative change[1] (fold control) | |
|---|---|---|---|
| | | LPS- | LPS+ |
| Whole cell | 100 | 4.2±0.5[2] | 1.3±0.6 |
| Cell wall | 100 | 8.3±1.4 | 2.1±0.7 |
| Peptidoglycans | 10 | 11.2±2.1 | 3.5±0.8 |
| | 50 | 14.5±2.7 | 4.7±1.1 |
| | 100 | 18.3±1.2 | 6.3±1.3 |

[1]RAW 264.7 cells ($5\times10^5$ cells/mL) were cultured at 37°C in 5% $CO_2$ with various samples under unstimulated (LPS-) or 1 μg/mL of LPS-stimulated (LPS+) conditions, and IL-6 production in culture supernatants was assessed by ELISA.
[2]Results are expressed as the mean's of fold increase for triplicate experiments.

activated immune cells and their role in host defenses and overall health. PG from *L. plantarum* PS-21 is expected to be proven as a safe immunopotentiating factor useful in maintaining the host immunity and to be developed as an important food additive.

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