

## ***In Vitro* Immunopotentiating Activities of Cellular Fractions of Lactic Acid Bacteria Isolated from *Kimchi* and Bifidobacteria**

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**Abstract** The present study represents the investigation of *in vitro* immunopotentiating activities of cellular fractions of major lactic acid bacteria found in kimchi (KLAB) and bifidobacteria. The macrophage cells, RAW264.7, were stimulated with heat-killed whole-cell, cell-wall, and cytoplasmic fractions of four strains of KLAB (*Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Lactobacillus plantarum*, and *Lactobacillus sake*) and two strains of bifidobacteria (*Bifidobacterium longum* and *Bifidobacterium lactis*) each, and then the production of nitric oxide (NO) and cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) was measured by Griess and ELISA assays, respectively. Heat-killed whole-cell and cell-wall fractions - but not the cytoplasmic fraction - from all strains of KLAB significantly increased the production of NO in RAW264.7 cells, and all fractions from bifidobacteria exerted similar effects. In the production of TNF- $\alpha$ , heat-killed whole-cell and cell-wall fractions of *L. plantarum* showed the strongest effect, followed by *L. sake* and *B. lactis*, whereas other KLAB fractions did not exert any effect. In the production of IL-6, only whole-cell and cell-wall fractions of *L. plantarum* were effective. These results, taken together, indicate that *L. plantarum* might play a critical role in the immunopotentiating activities of *kimchi*.

**Key words:** *Kimchi* lactic acid bacteria, bifidobacteria, immunopotentiating activities, heat-killed whole cells, cell wall, cytoplasm

Some lactic acid bacteria (LAB), called probiotics, are found to offer several health benefits including modulation of immune responses, balancing of colonic microbiota, and

reduction of fecal enzymes implicated in cancer development to humans [2, 3, 6, 7, 21]. Components and products of these microorganisms can activate macrophages to kill tumors [5]. In particular, the activation of macrophages by the cell-wall fraction of LAB results in the production of cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukins (e.g., IL-6) and the expression of inducible nitric oxide synthase, which may be responsible for the cytotoxicity of macrophages against tumor cells, either directly or indirectly [5, 18]. Tejada-Simon and Pestka [29] showed that cell-wall and cytoplasmic extracts of LAB stimulated the production of proinflammatory cytokine and nitric oxide (NO) in murine macrophages. We recently reported that the cytoplasmic fraction of *Lactococcus lactis* ssp. *lactis* has immunopotentiating activity in spleen, peritoneal exudates, and natural killer cells of mice [13].

*Kimchi* is a group of Korean traditional salted and fermented foods prepared with several kinds of vegetables such as cabbage, radish, and onion; spices such as garlic, red pepper, and ginger; and fish juice [20]. Many LAB are involved in the fermentation of *kimchi*, and we previously isolated four strains of LAB in *kimchi* (KLAB) - *Leuconostoc mesenteroides* (*Leuc. mesenteroides*), *Leuconostoc citreum* (*Leuc. citreum*), *Lactobacillus plantarum* (*L. plantarum*), and *Lactobacillus sake* (*L. sake*) - from Baechu *kimchi* [14, 15]. Before ripening, *Leuc. mesenteroides* is the dominant microorganism in *kimchi*, whereas *L. plantarum* is a major organism in ripened *kimchi*. Recently, there was an attempt to enhance the beneficial health effects of *kimchi* by adding a new microbial strain [16]. Not only LAB but also bifidobacteria are known to possess several beneficial effects to health such as prevention of intestinal disorder, antibiotic action, treatment of liver damage, reduction of the risk of colon cancer, antitumor activity, and immunomodulatory effects [1, 8, 17, 19, 23]. However,

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until now the immunopotentiating activity of lactic acid bacteria originated from *kimchi* had not been investigated.

Although the immune-stimulating activities of LAB have been studied previously, there is little information on the immunopotentiating activities of KLAB. The present study investigated the immunopotentiating activities of cellular fractions of KLAB and bifidobacteria *in vitro*.

## MATERIALS AND METHODS

### Chemicals

TNF- $\alpha$ , IL-6, purified antibodies to TNF- $\alpha$  or IL-6 (rat anti-mouse), biotinylated rat anti-mouse TNF- $\alpha$  or IL-6, and tetramethylbenzidine were obtained from Pharmingen (San Diego, CA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Chagrin Falls, OH, U.S.A.). Other compounds were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

### Microbial Strains and Culture Conditions

All KLAB (*Leuc. mesenteroides*, *Leuc. citreum*, *L. plantarum*, and *L. sake*) and bifidobacteria (*B. longum* and *B. lactis*) were isolated as described previously [1, 2]. All strains were cultured in MRS broth media (Difco, Detroit, MI, U.S.A.) at 37°C for 24 h.

### Fractionation of Cellular Components

Cellular components of lactic acid bacteria were prepared as described previously [13]. After cultivation, all strains were harvested in a refrigerated centrifuge (Vision, Seoul, South Korea), washed three times with distilled water, and lyophilized for storage. The lyophilized cells were resuspended in distilled water at 10 mg/ml and sonicated with a cell disruptor (Sonics and Materials, Danbury, CT, U.S.A.) for 30 min in ice. The suspension was centrifuged at 800  $\times$ g for 30 min at 5°C, and the solid residue was

removed. Cell walls were then separated from the supernatant using an ultracentrifuge (Hitachi, Tokyo, Japan) at 70,000  $\times$ g for 30 min, with the supernatant and the precipitate corresponding to the cytoplasmic and cell-wall fractions, respectively (Fig. 1).

### Cell Culture

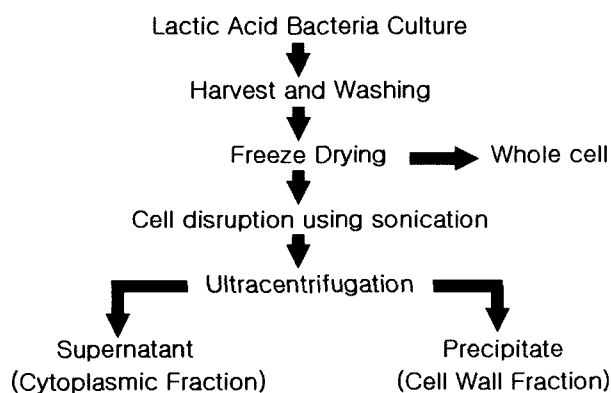
Cells from the RAW264.7 murine macrophage cell line were obtained from the Korean Cell Line Bank (Seoul, South Korea) and grown in DMEM media without phenol red supplemented with 10% (v/v) FBS, penicillin (100  $\mu$ g/ml), streptomycin (100 U/ml), and sodium bicarbonate (3.7 mg/ml) in a 5% CO<sub>2</sub> incubator (Forma Scientific, Marietta, OH, U.S.A.) at 37°C. Macrophage cells were plated in 96-well plates at  $5 \times 10^4$  cells/well, precultured for 24 h, and then used for experiments.

### Determination of Nitrite Production

NO production of macrophage cell was determined using nitrite measurements, which is the metabolite of NO oxidation [4]. After cultivation, cultured medium was mixed with an equal volume of Griess reagent (1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in 96-well plates. After the mixed solution had reacted for 5 min, its absorbance at 450 nm was measured using an ELISA reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Each set of experiments was performed at least three times. All results are presented as mean  $\pm$  standard deviation.

### Measurement of TNF- $\alpha$ and IL-6

The cytokine production of macrophages stimulated with the cellular components of the KLAB and bifidobacteria was measured using an ELISA kit (Pharmingen) as described previously [13]. Briefly, microtiter plates were coated overnight at 4°C with 50  $\mu$ l per well of purified rat antimouse cytokine (TNF- $\alpha$  and IL-6)-detecting antibody (Pharmingen) in 0.1 M sodium bicarbonate buffer, pH 8.2. Plates were washed three times with phosphate-buffered saline (PBS) containing 0.2% Tween-20 (PBS-T), blocked with 100  $\mu$ l of 3% (w/v) bovine serum albumin (BSA) in PBS at 37°C for 30 min, and then washed three times with PBS-T. Standard murine cytokines or samples were diluted in PBS-T solution containing 1% BSA, and 50  $\mu$ l aliquots were added to the wells. Plates were incubated at 4°C overnight and washed four times with PBS-T. Fifty  $\mu$ l of the monoclonal biotinylated rat antimouse antibody for the detection of each of the cytokines tested (Pharmingen), which was diluted in BSA-PBS, was added to each well. Plates were incubated at room temperature for 60 min and washed six times with PBS-T. Fifty  $\mu$ l of streptavidin-alkaline phosphatase conjugate diluted in BSA-PBS was added to each well. The plates were incubated at room temperature for 30 min and then washed with PBS-T,



**Fig. 1.** Scheme of fractionation of whole cell, cell wall, and cytoplasm from *kimchi* lactic acid bacteria and bifidobacteria.

after which 50  $\mu$ l of the substrate was added to each well. The absorbance at 405 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, U.S.A.), and cytokine concentrations were determined using the calibration curve. Each set of experiments was repeated at least three times. All results are presented as mean $\pm$ standard deviation.

### RESULTS AND DISCUSSION

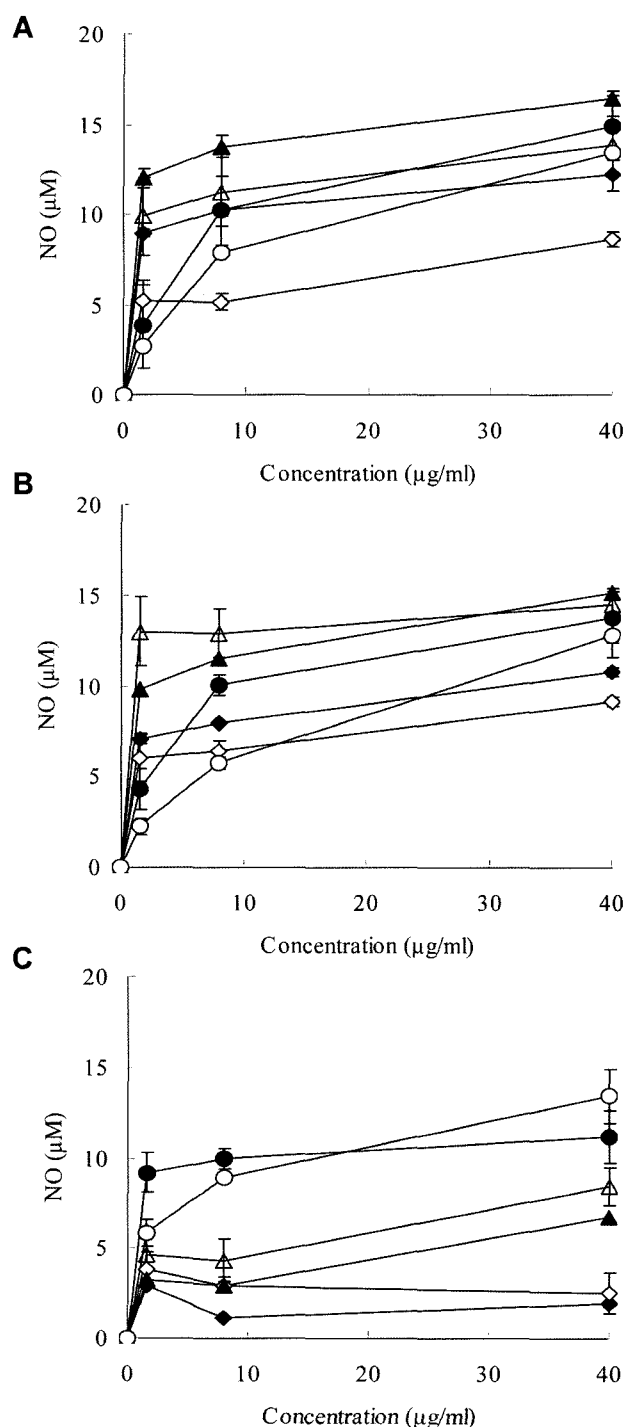
As shown in Fig. 2, whole-cell and cell-wall fractions of KLAB exhibited significantly increased production of NO in RAW264.7 cells, whereas the cytoplasmic fraction of KLAB exhibited relatively low activity. All the fractions of bifidobacteria exerted a similar effect. Among the four strains from kimchi, *L. plantarum* showed the highest activity, which was superior to that of bifidobacteria.

*L. plantarum* also showed stronger activities than other KLAB and bifidobacteria in the production of TNF- $\alpha$  and IL-6 (Figs. 3 and 4). In the production of TNF- $\alpha$ , whole-cell and cell-wall fractions of *L. plantarum* exerted the strongest effect, following by *L. sake* and *B. lactis*, whereas the other KLAB fractions did not show any effect (Fig. 3). Whole-cell and cell-wall fractions of *L. plantarum* were also the most effective in producing IL-6, and *L. sake* and *B. lactis* exerted only slight effects. These results indicate that *L. plantarum* plays a key role in the immunopotentiating activities of kimchi.

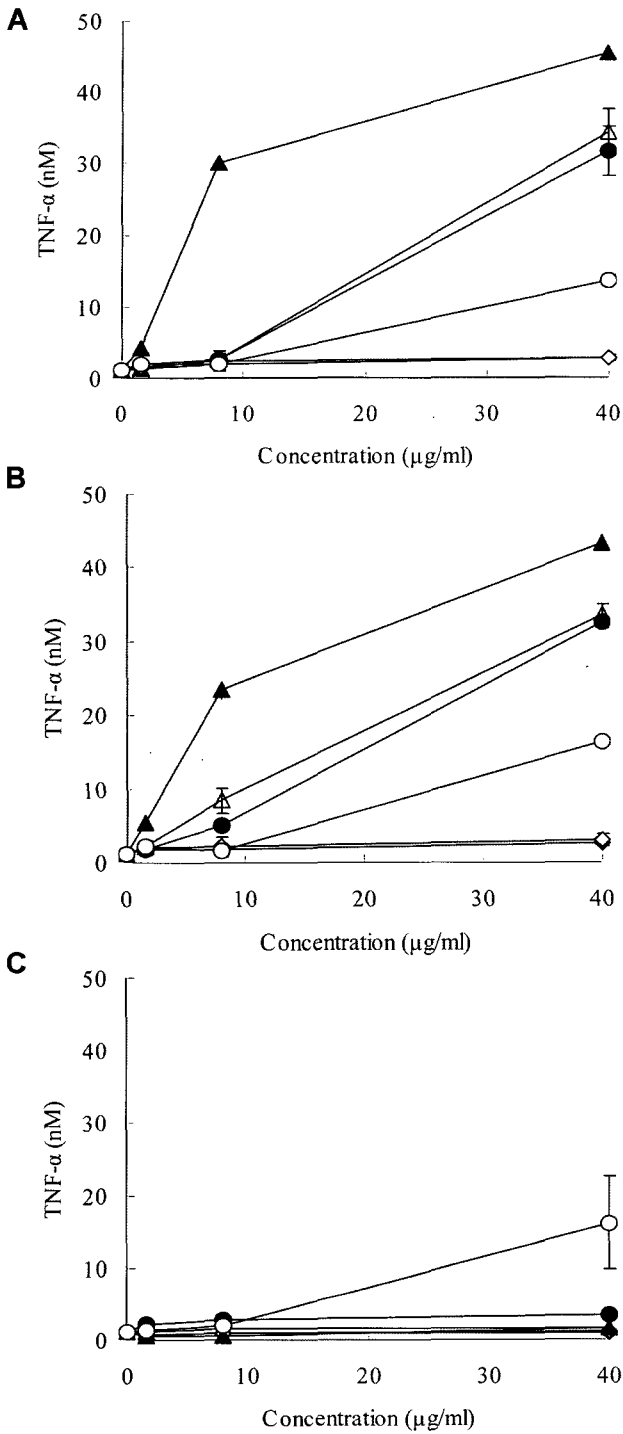
There is accumulating evidence that some LAB offer several health benefits including enhancing the immune responses, reducing fecal enzymes implicated in cancer initiation, treatment of diarrhea associated with travel and antibiotic therapy, and prevention of ulcers related to *Helicobacter pylori* [9]. It is known that LAB such as lactobacilli and bifidobacteria subspecies inhibit the development of colorectal cancer [26], and it has been suggested that the antitumor activities of LAB are attributable to the enhancement of the host's immune response, quantitative and/or qualitative alterations in the intestinal microflora incriminated in producing putative carcinogen and promoters, production of antitumorigenic or antimutagenic compounds in the colon, alteration of the metabolic activities of intestinal microflora [7, 25, 27], and direct suppression of cancer cells [12–14, 24].

Macrophages play a prominent role in host defenses by inducing cellular damage in infectious agents and tumor mediation. Activated macrophages are able to recognize and lyse tumor cells that are resistant to cytostatic drugs and can play a key role in novel immunotherapeutic cancer treatments. In the presence of pathogen infection and tumor development, macrophages produce NO to kill the antigen, and secrete IL-6 and cytokines such as TNF- $\alpha$  that activate specific immune cells. NO is known to affect the production

of several cytokines containing IL-6 and TNF- $\alpha$ , which are major cytokines involved in destroying infectious pathogens and tumor cells [18].

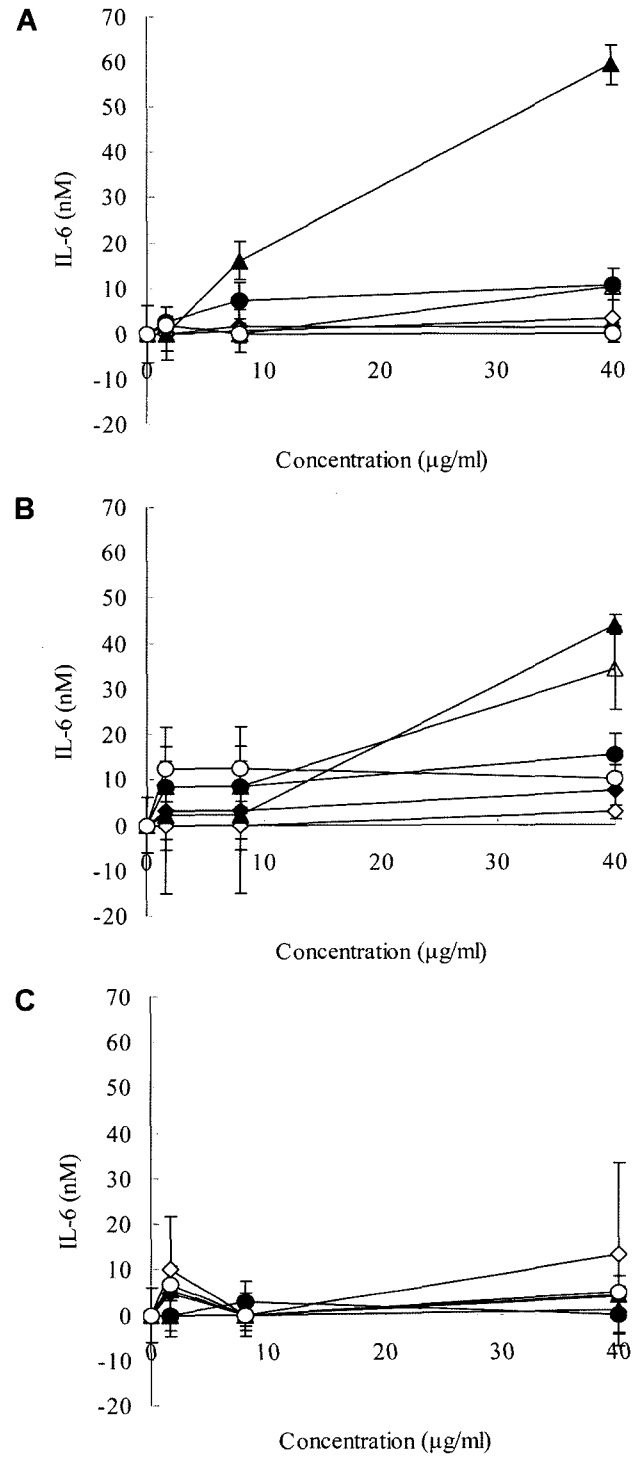


**Fig. 2.** Production of NO in macrophages treated with heat-killed whole-cell (A), cell-wall (B), and cytoplasmic (C) fractions at concentrations of 0–40  $\mu$ g/ml. *Leuc. mesenteriodes* (◆); *Leuc. citreum* (◇); *L. plantarum* (▲); *L. sake* (△); *B. longum* (●); *B. lactis* (○). Each set of experiments was performed at least three times.



**Fig. 3.** Production of TNF- $\alpha$  in macrophages treated with heat-killed whole-cell (A), cell-wall (B), and cytoplasmic (C) fractions at concentrations of 0–40  $\mu\text{g/ml}$ . *Leuc. mesenteroides* (◆); *Leuc. citreum* (◇); *L. plantarum* (▲); *L. sake* (△); *B. longum* (●); *B. lactis* (○). Each set of experiments was performed at least three times

Many LAB participate in the fermentation of *kimchi*: before ripening, *Leuc. mesenteroides* is the dominant microorganism, whereas *L. plantarum* is a major organism



**Fig. 4.** Production of IL-6 in macrophages treated with heat-killed whole-cell (A), cell-wall (B), and cytoplasmic (C) fractions at concentrations of 0–40  $\mu\text{g/ml}$ . *Leuc. mesenteroides* (◆); *Leuc. citreum* (◇); *L. plantarum* (▲); *L. sake* (△); *B. longum* (●); *B. lactis* (○). Each set of experiments was performed at least three times.

in ripened *kimchi*. The present study demonstrated that cellular fractions of some KLAB stimulated macrophages

to produce NO, IL-6, and TNF- $\alpha$ , thereby indicating their immunopotentiating activities. A study has shown that peptidoglycan, a cell-wall component of Gram-positive bacteria, stimulated macrophages to produce NO and cytokines such as TNF- $\alpha$ , interferon- $\gamma$ , and interleukins [29]. Consistent with these results, our results also showed that activation of macrophages stimulated with KLAB is mainly due to the cell-wall fraction (Figs. 2–4). We have previously shown that the cytoplasmic fraction of *L. lactis* ssp. *lactis* exerts immunopotentiating activity in spleen, peritoneal exudates, and natural killer cells of mice [13]. The present study also showed that all fractions of bifidobacteria exerted similar effects in the production of NO and TNF- $\alpha$  (Figs. 2 and 3). These results indicate that the immunopotentiating activities of LAB are also attributed to the cytoplasmic components of LAB.

In the present study, *L. plantarum* not only showed the strongest immunopotentiating activity among the four strains isolated from *kimchi*, but it was also even superior to that of bifidobacteria. These results suggest that *L. plantarum* is the major strain contributing to the health-promoting activity of *kimchi*. It is well-known that garlic, red pepper, and cabbage (all ingredients of *kimchi*) contain a high level of phytochemicals with chemopreventive potential [22, 28]. Glycoprotein, a product of *L. plantarum* KLAB21 isolated from *kimchi*, also showed antimutagenic activity [3]. Thus, *kimchi*, which is rich in LAB and other phytochemicals, is considered to be a health-benefiting food.

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