J. Microbiol. Biotechnol. (2006), **16**(5), 786–790

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

In Vivo Immunopotentiating Effects of Cellular Components from *Lactococcus lactis* ssp. *lactis*

KIM, JI YEON[1], SEONGKYU LEE[2], DO-WON JEONG[3], SATOSHI HACHIMURA[2], SHUICHI KAMINOGAWA[4], AND HYONG JOO LEE[3]*

[1]*Department of Food Standard Evaluation, Korea Food & Drug Administration, Seoul 122-704, Korea*
[2]*Department of Applied Biological Chemistry, The University of Tokyo, Tokyo, Japan*
[3]*School of Agricultural Biotechnology, and Center of Agricultural Biomaterials, Seoul National University, Seoul 151-742, Korea*
[4]*Department of Food Science and Technology, College of Bioresource Science, Nihon University, Tokyo, Japan*

Received: February 23, 2005
Accepted: April 19, 2005

Abstract Cellular components of *Lactococcus lactis* ssp. *lactis* (heat-killed whole cells, cytoplasm, and cell walls) were tested for their *in vivo* immunopotentiating activity. Peritoneal macrophages from mice orally administered with heat-killed whole cells exhibited significantly greater phagocytic activity than the groups administered with cell-wall fraction or cytoplasm fraction. The cytotoxicity of natural-killer cells was the highest in the group administered with whole cells, and the production of cytokines (IFN-γ, IL-2, and IL-12) in spleen cells was significantly higher, when cellular components were injected, and it tended to be higher in the cell-wall and cytoplasm groups than in the whole-cell group. Interestingly, the cytokine production of Peyer's patch cells was high, when cytoplasm fractions were administered. These results demonstrate that whole cells and cytoplasm and cell-wall fractions of *L. lactis* ssp. *lactis* have immunopotentiating activities, which are related to the stimulation of Peyer's patches.

Key words: Cytokine, immunopotentiating activity, *Lactococcus lactis* ssp. *lactis*, NK cell activity, phagocytic activity

The capacity of certain lactic acid bacteria (LAB) to function as probiotics, when ingested as a part of fermented dairy products or dietary adjuncts, is receiving increasing attention, and an extensive volume of literature on the possible health benefits associated with the consumption of LAB is now available [1, 5, 11, 13, 15]. The mechanisms underlying these favorable effects include changes in viable populations of microorganisms in the intestinal flora, competition for adhesion sites and nutrients between ingested bacteria and potential pathogens, production of antibacterial substances, and stimulation of the immune system. With regards immunity, Perdigon *et al.* [17] observed enhanced macrophage and lymphocyte activities in mice after administration of a mixed culture of *Lactobacillus acidophilus* and *Lb. casei*, and also reported that peritoneal macrophages in mice were activated by the oral administration of *Lb. casei* and *Lb. bulgaricus* [16, 18]. Similar results have been found for *Streptococcus thermophilus* and *Lb. acidophilus* orally administered [19], and heat-killed *Lb. casei* injected into mice [20]. The oral administration of LAB and fermented milk has been demonstrated to increase mitogenic responses [3]. These reports indicate that orally administered LAB and fermented milk stimulate the host's immune system. However, most reports on the immunopotentiating activity of LAB have focused on whole LAB cells and their peptidoglycans, with little attention being paid to the soluble fraction, although the potential in food applications is different between soluble and insoluble materials. We have recently described the *in vitro* immunopotentiating activity of the cellular component, containing the soluble fraction of *Lactococcus lactis* [12]. Those results indicated that *L. lactis* cytoplasm and cell-wall fractions as well as whole cells are capable of stimulating lymphocytes and macrophages to produce several cytokines. Based on these observations, we demonstrated the systemic and mucosal immune responses of mice administered orally with whole cells and cell-wall and cytoplasm fractions of *L. lactis* ssp. *lactis*.

L. lactis was cultured in M17 media (Difco, Detroit, MI, U.S.A.) for 18 h at 30°C. After cultivation, the cells 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

*Corresponding author
Phone: 82-2-880-4853; Fax: 82-2-873-5095;
E-mail: leehyjo@snu.ac.kr

at 10 mg/ml in distilled water and sonicated with a cell disruptor (Sonics and Materials, Danbury, CT, U.S.A.) for 30 min on ice. After the suspension was centrifuged at 800 ×*g* for 30 min at 5°C, the pellet was removed. A cell-wall fraction of the pellet and a cytoplasm fraction of the supernatant were obtained from the supernatant using an ultracentrifuge (Hitachi, Tokyo, Japan) at 70,000 ×*g* for 30 min.

To elucidate the immunopotentiating activity of the cellular components of *L. lactis*, whole cells, cell walls, or cytoplasm of *L. lactis* were administered to mice, and the phagocytic function, natural killer (NK) cell activity, and cytokine production were assayed. Oral administration was performed as follows. Six-week-old male BALB/c mice (Clea Japan, Tokyo, Japan) were orally given the desired dose of cellular components dissolved in phosphate-buffered saline (PBS). *L. lactis* cellular components were administered orally for 7 consecutive days at 4 mg/mouse/day, with the solution delivered to the stomach via a stainless steel needle. PBS was used for controls. Each mouse was killed the day after completing administration, and then peritoneal exudate cells (PEC), spleen, and Peyer's patch cells were isolated.

Phagocytic cells are the major effectors of natural immunity, and there are numerous reports on the relationship between LAB and natural immunity [4]. The phagocytic activity of PEC was evaluated in a culture with fluorescent microparticles [23]. Therefore, PEC was isolated from the peritoneal cavity of the mice by lavage with 5 ml of HBSS after the oral injection of the cellular component. After centrifugation, the cell pellet was washed twice with HBSS and resuspended in 1 ml of HBSS-HEPES. Twenty μl of Fluoresbrite carboxylate microspheres (2.0 μm; Polyscience, Warrington, PA, U.S.A.) diluted 100-fold with HBSS-HEPES was added to the PEC suspension, which was then incubated for 1 h at 37°C. After stopping the reaction by adding 2 ml of cold EDTA-PBS and collecting a cell pellet by centrifugation, the pellet was resuspended in 300 μl of EDTA-PBS, and the phagocytic activity was measured using flow cytometry. Figure 1 shows the phagocytic activity of peritoneal macrophages as the phagocytic uptake of fluorescent microparticles by PEC, when orally administered. The microparticles incorporated into cells were counted with a flow cytometer (Fig. 1A), and the results are expressed as the percentages of cells in which one, two, or more than two particles were incorporated (Fig. 1B). The phagocytic activity of peritoneal macrophages was significantly greater in mice administered with heat-killed whole cells than in the PBS, cell-wall, and cytoplasm groups (Fig. 1): For example, the activity was about 1.5 times higher than in the PBS group.

NK activity, another effector of natural immunity, of spleen cells was determined using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, U.S.A.). The CytoTox 96 assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants was then measured with an enzymatic assay. Briefly, the isolated spleen cells were used as effector cells, and cells from the mouse Moloney leukemia cell line, Yac-1, were used as the target cells. Yac-1 cells (2×10^4 cells/ml) in RPMI-1640 without phenol red were seeded in round-bottomed tissue culture plates. Subsequently, 0.1 ml

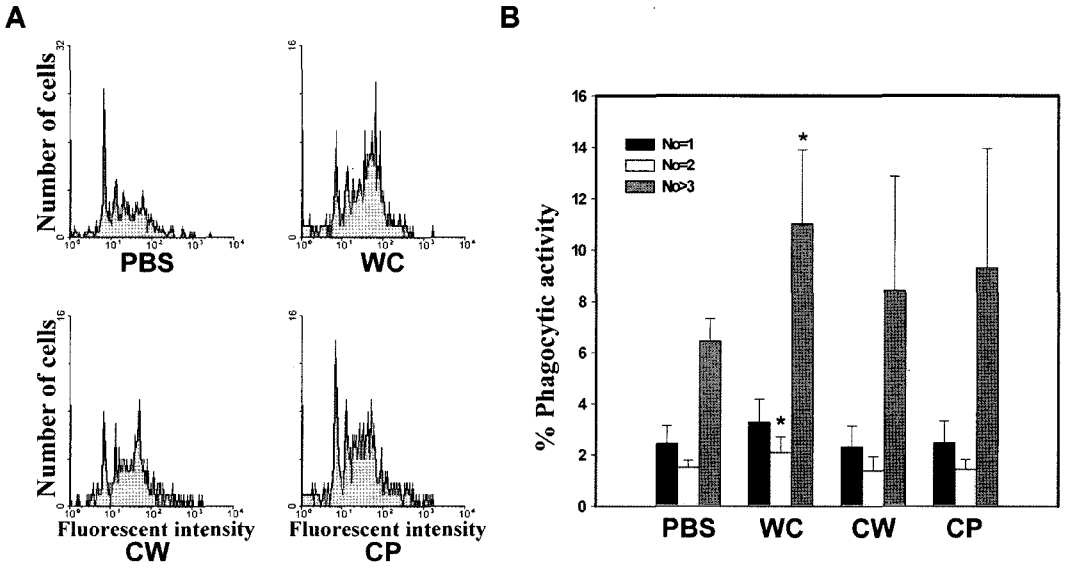

Fig. 1. Phagocytic activity of PEC obtained from mice orally injected with whole cell (WC), cell-wall (CW), and cytoplasm (CP) fractions of *L. lactis* at 4 mg/mouse for 7 consecutive days.

On day 7, the phagocytic activity was measured using flow cytometry. **A**. Typical result of flow cytometry. **B**. Analysis of PEC phagocytic activity based on flow cytometry ("No" is the number of microparticles taken up by PEC). Data are shown as mean±SD values (n=6, *P<0.05).

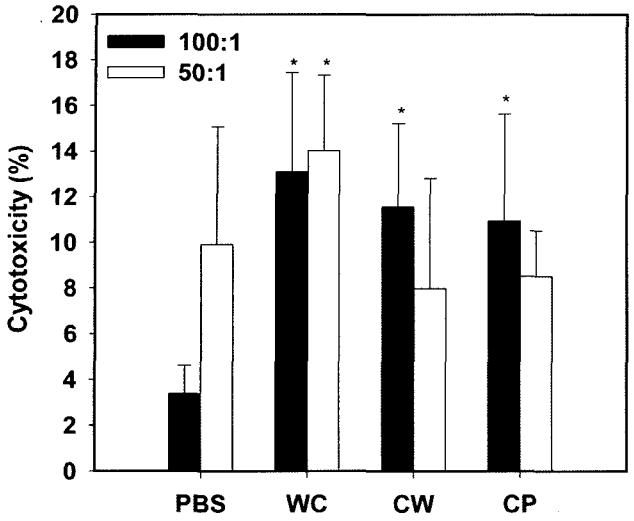

Fig. 2. Percentage cytotoxicity of NK cells in spleen cells obtained from mice orally administered with whole cell (WC), cell-wall (CW), and cytoplasm (CP) of *L. lactis* against Yac-1 cells; effector:target cell ratios were 100:1 and 50:1.
The cytotoxicity was measured using LDH assays. Data are shown as mean±SD values (n=6, *P<0.05).

of the spleen cells suspension treated with cellular components was added at appropriate concentrations. The assay plates were incubated for 4 h in a humidified chamber at 37°C and 5% CO_2 and then centrifuged at 250 ×g for 4 min. The supernatants were transferred, and the substrate mix was added to each well. After incubation for 30 min, absorbance at 490 nm was measured. The NK-cell activity was calculated as a percentage of effector-cell-specific lysis. The percentage of specific LDH release was calculated according to the following formula: Specific lysis (%)= (experimental-effector spontaneous-target spontaneous)/ (target maximum-target spontaneous)×100. Enhanced NK cytotoxicity has been reported to be effective in cancer prevention. These cells may play an important role in the regulation of tumor development and metastasis [10]. The primary target of LAB for their immunostimulatory effect was shown to be NK cells [6], and NK cell activation by *Lb. casei* has also been reported [9, 21]. However, in the present study, all mice receiving *L. lactis* cellular fractions showed NK activities similar to those in control mice (Fig. 2), and the group treated with heat-killed whole cells tended to have a slightly higher cytotoxic activity than the controls.

The production of cytokines by spleen and Peyer's patch cells in response to Con A (5 μg/ml) or LPS (20 ng/ml) for 48 h at 37°C was assessed using a sandwich ELISA: Con A was used for measuring IFN-γ, IL-2, and IL-4, and LPS was used for IL-12. Thus, cell-free supernatants

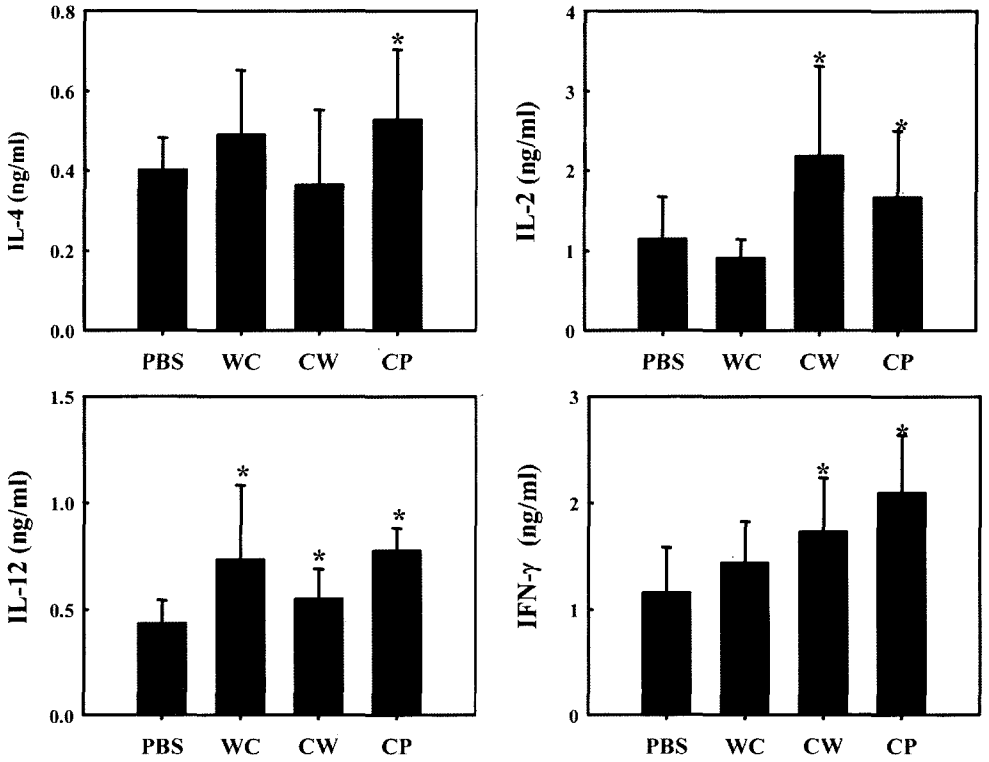

Fig. 3. Production of cytokines by spleen cells from mice orally administered with whole cell (WC), cell-wall (CW) and cytoplasm (CP) fractions of *L. lactis*.
Spleen cells were cultured with mitogen (Con A for IFN-γ, IL-2, and IL-4; or LPS for IL-12) for 48 h. The concentrations of cytokines in culture supernatant were measured using ELISA, and the data are shown as mean±SD values (n=6, *P<0.05).

were harvested and stored at −20°C until assayed. Briefly, microtiter plates were coated overnight at 4°C with purified rat anti-mouse cytokine-capture antibody at 50 μl/well (Pharmingen, San Diego, CA, U.S.A.) in 0.1 M sodium bicarbonate buffer (pH 8.2). The plates were washed three times with PBS containing 0.2% Tween-20 (PBS-T). Plates were blocked with 100 μl of 1% (w/v) bovine serum albumin (BSA) in PBS for 30 min at 37°C and washed three times with PBS-T. Standard murine cytokines or samples were diluted in PBS-T solution containing 1% BSA, and 50-μl aliquots were added to the appropriate wells. The plates were incubated overnight at 4°C, washed four times with PBS-T, and then 50 μl of biotinylated rat anti-mouse cytokine-capture monoclonal antibody 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 μl of streptavidin-alkaline-phosphatase conjugate diluted in BSA-PBS was added to each well, and plates were incubated for 30 min at room temperature. The plates were then washed with PBS-T, and 50 μl of substrate (*p*-nitrophenylphosphate) was added to each well. The absorbance was read at 405 nm on a microplate reader (Bio-Rad, Hercules, CA, U.S.A.), and cytokine concentrations were quantified using a standard curve. As seen in Fig. 3, the production of IL-2, IL-12, and IFN-γ was higher in spleen cells when orally administered with either cytoplasm or cell-wall fractions than with whole cells. The production of cytokines in Peyer's patches was also studied. Interestingly, in Peyer's patch cells, the cytoplasm fraction was more effective than whole cells and the cell-wall fraction: The production of IL-2, IL-12, and IFN-γ by Peyer's patch cells was significantly higher in mice orally administered with cytoplasm fractions than in the other groups (Fig. 4). Macrophages are the main producers of IL-12, IL-6, and TNF-α, and they are important target cells for the antitumor or immunomodulating effects of some microorganisms. In particular, IL-12 potently stimulates cytotoxic T cells and NK cells and enhances the production of several cytokines, such as IFN-γ, IL-2, and TNF-α. In the present study, the administration of cellular components of *L. lactis* augmented mainly the production of IFN-γ, IL-2, and IL-12, which is consistent with earlier studies [2, 7, 8, 14, 22]. The results demonstrate that *L. lactis* whole cells as well as their cytoplasm and cell-wall fractions have immunostimulating activities, and that this mechanism is related to the stimulation of Peyer's patches. The reason why the cytoplasm fraction was so effective in stimulating Peyer's patch cells is not clear, but it might have been due to the fact that this fraction is effectively incorporated into the Peyer's patch follicles.

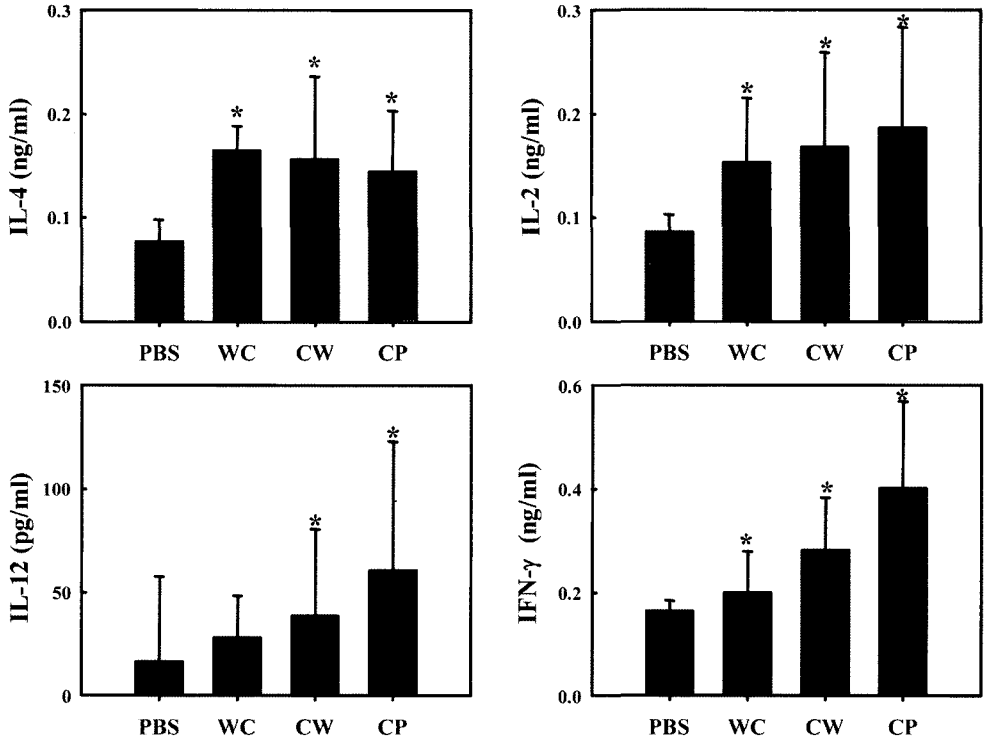

Fig. 4. Production of cytokines by Peyer's patch cells from mice orally administered with whole cell (WC), cell-wall (CW) and cytoplasm (CP) fractions of *L. lactis*.

Peyer's patch cells were cultured with mitogen (Con A for IFN-γ, IL-2, and IL-4; or LPS for IL-12) for 48 h. The concentrations of cytokines in the culture supernatant were measured using ELISA, and the data are shown as mean±SD values (n=6, *P<0.05).

In conclusion, the present results suggest that *L. lactis* whole cells as well as their cytoplasm and cell-wall fractions are capable of stimulating splenocytes, Peyer's patches, and macrophages to produce several cytokines. Elevated cytokine production (particularly of IFN-γ and IL-12) was correlated with NK cell activity, which is important in the understanding of the mechanisms underlying the immunoregulatory function of *L. lactis* and its potential applications.

REFERENCES

1. Bae, E.-A., N.-Y. Kim, M. J. Han, M.-K. Choo, and D.-H. Kim. 2003. Transformation of ginsenosides to compound K (IH-901) by lactic acid bacteria of human intestine. *J. Microbiol. Biotechnol.* **13:** 9–14.
2. Cross, M. L., R. R. Mortensen, J. Kudsk, and H. S. Gill. 2002. Dietary intake of *Lactobacillus rhamnosus* HN001 enhances production of both Th1 and Th2 cytokines in antigen-primed mice. *Med. Microbiol. Immunol.* **191:** 49–53.
3. De Simone, C., R. Vesely, R. Negri, S. B. Bianchi, S. Zanzoglu, A. Cilli, and L. Lucci. 1987. Enhancement of immune response of murine Peyer's patches by a diet supplemented with yogurt. *Immunopharmacol. Immunotoxicol.* **9:** 87–100.
4. Gill, H. S., K. J. Rutherfurd, J. Prasad, and P. K. Gopal. 2000. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *Br. J. Nutr.* **83:** 167–176.
5. Gilliland, S. E. 1990. Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiol. Rev.* **7:** 175–188.
6. Haller, D., S. Blum, C. Bode, W. Hammes, and E. J. Schiffrin. 2000. Activation of human peripheral blood mononuclear cells by nonpathogenic bacteria *in vitro*: Evidence of NK cells as primary targets. *Infect. Immun.* **68:** 752–759.
7. Hessle, C., L. A. Hanson, and A. E. Wold. 1999. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. *Clin. Exp. Immunol.* **116:** 276–282.
8. Kato, I., K. Tanaka, and T. Yokokura. 1999. Lactic acid bacterium potently induces the production of interleukin-12 and interferon-γ by mouse splenocytes. *Int. J. Immunopharmacol.* **21:** 121–131.
9. Kato, I., T. Yokokura, and M. Mutai. 1984. Augmentation of mouse natural killer cell activity by *Lactobacillus casei* and its surface antigens. *Microbiol. Immunol.* **28:** 209–217.
10. Kelly, J. M., P. K. Darcy, J. L. Markby, D. I. Godfrey, K. Takeda, H. Yagita, and M. J. Smyth. 2002. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. *Nature Immunol.* **3:** 83–90.
11. Kim, H.-J., J.-H. Kim, J. H. Son, H.-J. Seo, S.-J. Park, N.-S. Paek, and S.-K. Kim. 2003. Characterization of bacteriocin produced by *Lactobacillus bulgaricus*. *J. Microbiol. Biotechnol.* **14:** 503–508.
12. Kim, J. Y., S. Lee, S. Hachimura, S. Kaminogawa, and H. J. Lee. 2003. *In vitro* immunopotentiating activity of cellular component of *Lactococcus lactis* ssp. *lactis*. *J. Microbiol. Biotechnol.* **13:** 202–206.
13. Lee, H.-Y., J.-H. Park, S.-H. Seok, S.-A. Cho, M.-W. Baek, D.-J. Kim, Y.-H. Lee, and J.-H. Park. 2004. Dietary intake of various lactic acid bacteria suppresses type 2 helper T cell production in antigen-primed mice splenocyte. *J. Microbiol. Biotechnol.* **14:** 167–170.
14. Murosaki, S., K. Muroyama, Y. Yamamoto, and Y. Yoshikai. 2000. Antitumor effect of heat-killed *Lactobacillus plantarum* L-137 through restoration of impaired interleukin-12 production in tumor-bearing mice. *Cancer Immunol. Immunother.* **49:** 157–164.
15. Ouwehand, A. C., S. Salminen, and E. Isolauri. 2002. Probiotics: An overview of beneficial effects. *Antonie van Leeuwenhoek* **82:** 279–289.
16. Perdigon, G., S. Alvarez, and H. A. P. de Ruiz. 1991. Immunoadjuvant activity of oral *Lactobacillus casei*: Influence of dose on the secretory immune response and protective capacity in intestinal infections. *J. Dairy Res.* **58:** 485–496.
17. Perdigon, G., M. E. N. de Macias, S. Alvarez, G. Oliver, and H. A. P. de Ruiz. 1986. Effect of orally administered lactobacilli on macrophage activation in mice. *Infect. Immun.* **53:** 404–410.
18. Perdigon, G., M. E. N. de Macias, S. Alvarez, G. Oliver, and H. A. P. De Ruiz. 1990. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. *J. Dairy Sci.* **57:** 255–264.
19. Perdigon, G., M. E. Nader de Macias, S. Alvarez, M. Medici, G. Oliver, and H. A. A. Pesce de Ruiz. 1987. Enhancement of immune response in mice fed with *Streptococcus thermophilus* and *Lactobacillus acidophilus*. *J. Dairy Sci.* **70:** 919–926.
20. Saito, H., H. Tomioka, and K. Nagashima. 1987. Protective and therapeutic efficacy of *Lactobacillus casei* against experimental murine infections due to *Mycobacterium fortuitum* complex. *J. Gen. Microbiol.* **133:** 2843–2851.
21. Takagi, A., T. Matsuzaki, M. Sato, K. Nomoto, M. Morotomi, and T. Yokokura. 2001. Enhancement of natural killer cytotoxicity delayed murine carcinogenesis by a probiotic microorganism. *Carcinogenesis* **22:** 599–605.
22. Yasutake, N., T. Matsuzaki, K. Kimura, S. Hashimoto, T. Yokokura, and Y. Yoshikai. 1999. The role of tumor necrosis factor (TNF)-α in the antitumor effect of intrapleural injection of *Lactobacillis casei* strain Shirota in mice. *Med. Microbiol. Immunol.* **188:** 9–14.
23. Yoshizawa, Y., J. Tsunehiro, K. Nomura, M. Itoh, F. Fukui, A. Ametani, and S. Kaminogawa. 1996. *In vivo* macrophage-stimulation activity of the enzyme-degraded water-soluble polysaccharide fraction from a marine alga (*Gracilaria verrucosa*). *Biosci. Biotechnol. Biochem.* **60:** 1667–1671.