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In Vitro Immunopotentiating Activity of Cellular Components of *Lactococcus lactis* ssp. *lactis*

KIM, JI YEON[1], SEONGKYU LEE[2], SATOSHI HACHIMURA[2], SHUICHI KAMINOGAWA[2], AND HYONG JOO LEE[1]*

[1]*Department of Food Science and Technology, School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea*
[2]*Department of Applied Biological Chemistry, The University of Tokyo, Tokyo, Japan*

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Abstract To determine the effect of immunopotentiating activity of cellular components of *Lactococcus lactis* ssp. *lactis*, the immune function was analyzed *in vitro* using mice cells. When stimulated with mitogens, productions of IFN-γ, IL-12, TNF-α, and IL-6 were enhanced in spleen cells treated with cellular components, with IL-4 production being the highest in spleen cells treated with cytoplasm fraction. Without mitogen stimulation, the productions of IFN-γ and IL-12 were the highest in spleen cells treated with heat-killed whole cell. TNF-α and IL-6 productions were also high in spleen cells treated with all cellular components. Only heat-killed whole cell showed significant enhancement in natural killer cell activity. In peritoneal exudates cells, TNF-α production was enhanced significantly by all cellular components of *Lactococcus lactis* ssp. *lactis*. These results indicate that the cellular components of *Lactococcus lactis* ssp. *lactis* are capable of stimulating immune cells to produce cytokines, and that both their cell walls and cytoplasm fraction contribute to these capacities.

Key words: Cell wall, cytoplasm, immune, *Lactococcus lactis* ssp. *lactis*

The capacity of certain lactic acid bacteria (LAB) to function as probiotics, when ingested as a part of fermented dairy products or as dietary adjuncts, has been receiving increasing attention, and an extensive body of literature addressing the possible health benefits associated with the consumption of LAB are available [3]. The mechanisms involved in the production of these favorable effects include changes in viable populations of microorganisms in the intestinal flora after ingestion, competition for adhesion sites and nutrients between the ingested bacteria and potential pathogens, production of antibacterial substances, and the action of these bacteria through the stimulation of the immune system. Related to immunity, Perdigon *et al.* [15] observed enhanced macrophage and lymphocyte activities in mice after administration of a mixed culture of *Lactobacillus acidophilus* and *L. casei*, and also reported activation of peritoneal macrophages in mice after oral administration of *L. casei* and *L. bulgaricus*. Similar results were also found with *Streptococcus thermophillus* and *L. acidophilus*, when orally delivered [16], and heat-killed *L. casei* administered to mice via injection [19]. However, most reports on the immunopotentiating activity of LAB, so far, have been focused on the whole cell of LAB or its peptidoglycans; little attention has been paid to the soluble fraction, although the potentiality of soluble materials for food applications differs from that of insoluble ones. In this study, the immunopotentiating effects of cellular components of *Lactococcus lactis* ssp. *lactis* used in dairy fermentation and cheese production were compared. The effects of *in vitro* exposure to whole cell, cell wall, and cytoplasm fraction on cytokine production and natural killer (NK) cell activity by mouse spleen cells and on TNF-α and IL-12 productions by mouse peritoneal exudate cells were determined.

*Corresponding author
Phone: 82-31-290-2585; Fax: 82-31-293-4789;
E-mail: leehyjo@snu.ac.kr

Materials and Methods

Experimental Animals

BALB/c male mice, 6 weeks old, purchased from Clea Japan Inc. (Tokyo, Japan), were housed in plastic cages in an air-conditioned room, and were given food and water *ad libitum*.

Fractionation of Cellular Components of *Lactococcus lactis* ssp. *lactis*

Lc. lactis was cultured in M17 media (Difco Laboratories, Detroit, MI, U.S.A.) for 18 h at 30°C. After cultivation, the cells were harvested in a refrigerated centrifuge (Vision Co.,

Seoul, 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 Inc., 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. Cell walls were then sedimented from the supernatant using ultracentrifuge (Hitachi, Tokyo, Japan) at 70,000 ×g for 30 min. The supernatant and the pellet correspond to the cytoplasm and the cell wall fractions, respectively.

Isolation of Spleen and Peritoneal Exudates Cells of Mice

Mice were sacrificed by cervical dislocation. Proteose peptone-induced macrophages from mice were isolated from the peritoneal cavity by lavage with 5 ml of HBSS 3 days after an intraperitoneal injection of 1.5 ml of autoclaved 10% proteose peptone (Difco Laboratories, Detroit, MI, U.S.A.). After centrifugation, the cell pellet was washed twice with HBSS and resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml of penicillin, and 100 μg/ml of streptomycin (RPMI-FCS). The cells were cultured at 2×10^6 cells/ml in 24-well plates. Spleens were removed aseptically, teased apart with tissue forceps in 10 ml RPMI-FCS, and centrifuged. Erythrocytes were lysed for 5 min on ice in 5 ml of a buffer containing 90% of 0.16 M ammonium chloride and 10% of 0.17 M Tris buffer (pH 7.2). Ten milliliters of fresh RPMI-FCS were added, and the cells were centrifuged, counted, and cultured to a final density of 5×10^6 cells/ml.

Analysis of Natural Killer Cell Activity

The CytoTox 96 Nonradioactive cytotoxicity assay kit (Promega, Madison, WI, U.S.A.) was used to assess NK cell activity of spleen cells. 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 measured with an enzymatic assay. Briefly, spleen cells were cultured, while being treated with the cellular fractions of *Lc. lactis* for 5 days at 50 μg/ml. Cultured spleen cells were used as effector cells. 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-bottom tissue culture plates. Subsequently, 0.1 ml of appropriate concentrations of the treated spleen cells was added. The assay plates were incubated for 4 h in a humidified chamber at 37°C and 5% CO_2, and the cultures were centrifuged at 250 ×g for 4 min. The supernatants were transferred, and the substrate mixture was added to each well. After incubation for 30 min, absorbances were measured at 490 nm. The NK cell activity was expressed as the percentage of effector cell-specific lysis. The percentage specific LDH release was calculated according to the formula:

Specific lysis (%)
=(experimental-effector spontaneous-target spontaneous)
/(target maximum-target spontaneous)×100

Quantification of Cytokine Production

Spleen and peritoneal exudate cells were added to each well of a 24-well plate and cultured with cellular fractions of *Lc. lactis* in the presence or absence of ConA (5 ng/ml) or LPS (20 ng/ml) for 48 h at 37°C. Cell-free supernatant fractions were harvested and stored at -20°C until assayed.

The presence of several cytokines in the culture supernatant was determined using a sandwich ELISA. Briefly, microtiter plates were coated overnight at 4°C with 50 μl per well of purified rat anti-mouse cytokine (IL-12, IFN-γ, TNF-α, IL-6, IL-2, or IL-4) capture antibody (Pharmingen, San Diego, CA, U.S.A.) in 0.1 M sodium bicarbonate buffer, pH 8.2. Plates were washed three times with PBS containing 0.2% Tween-20 (PBS-T). Plates were blocked with 100 μl of 3% (w/v) bovine serum albumin (BSA) in PBS at 37°C for 30 min 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 appropriate wells. Plates were incubated at 4°C overnight and washed four times with PBS-T. Fifty microliter of biotinylated rat anti-mouse cytokine detection monoclonal antibody for each respective cytokine (Pharmingen), which was diluted in BSA-PBS, were added to each well. Plates were incubated at room temperature for 60 min and washed six times with PBS-T. Fifty microliters of streptavidin-alkaline phosphatase conjugate diluted in BSA-PBS were added to each well. The plates were incubated at room temperature for 30 min and were then washed with PBS-T. Subsequently, 50 μl of the substrate was added to each well. Absorbances were read at 405 nm on a microplate reader (Bio-Rad, Hercules, CA, U.S.A.), and cytokine concentrations were quantified using the standard curve.

Statistics

Each set of experiments was done at least three times. All results were presented as mean±SD (standard deviation). Significant differences between the experimental and control groups were determined using Fisher's PLSD (Protected Least Significant Differences). Test values that resulted in $P<0.05$ were considered as significant.

Results and Discussion

Effect of *Lc. lactis* Cellular Fractions on the Cytokine Production in Spleen Cells Stimulated with Mitogens

T cells are the main effectors and regulators of cell-mediated immunities, and non-T cells such as antigen presenting cells regulate the response of T cells. Cytokines

Table 1. Effects of heat-killed whole cell, cell wall, and cytoplasm fraction of *Lc. lactis* on the production of cytokines in spleen cells stimulated with mitogens[d].

μg/ml	Fold control[c]					
	IFN-γ	TNF-α	IL-12	IL-6	IL-2	IL-4
WC[a] 10	1.6±0.2*	5.6±1.1*	1.4±0.2*	1.3±0.1*	-	-
50	1.9±0.1*	2.6±0.6*	1.9±0.1*	1.7±0.1*	-	-
100	2.0±0.2*	2.2±0.3	2.1±0.2*	2.5±0.1*	-	-
500	1.3±0.3*	6.0±2.2*	1.3±0.2*	1.0±0.1	15±5.6*	103±14*
CW[a] 10	1.5±0.1*	3.5±1.5*	1.2±0.1*	1.3±0.1*	-	-
50	2.0±0.1*	2.7±0.3	1.4±0*	1.4±0.1*	-	-
100	1.8±0.2*	8.6±3.3*	1.4±0.1*	1.8±0.2*	-	-
500	1.2±0.2	3.8±0.8*	1.1±0.1	0.9±0.1	75±13*	65±9.4*
CP[a] 10	1.5±0.1*	9.1±8.1*	1.3±0.1*	2.3±0.1*	-	-
50	1.3±0.1*	6.1±2.5*	1.4±0.1*	1.4±0*	-	193±80*
100	1.2±0.2*	4.0±0.4*	1.0±0.2	0.9±0.2	-	115±17*
500	1.7±0.2*	-[b]	1.1±0.1	1.0±0.1	4±6.5	287±40*

[a]WC, whole cell; CW, cell wall; CP, cytoplasm.
[b]-, not detected.
[c]Contents of cytokines were calculated by dividing experimental data by control values (n=3) and are averages of three separate cultures (*$P<0.05$, different from control by Fisher's PLSD).
[d]Mitogen used for the detection of IFN-γ, IL-2, and IL-4 was Con A (5 μg/ml) and that of TNF-α, IL-6, and IL-12 was LPS (10 μg/ml).

play a major role in both of these functions [1]. It was found that exposure of spleen cells to *Lc. lactis* cellular fractions affected cytokine productions. When stimulated with mitogen, production of several cytokines was significantly induced with all cellular components (Table 1). Among these, TNF-α production was the highest, but the production tended to decrease at 500 μg/ml. In contrast, IL-2 and IL-4 productions were high at the highest concentration (Table 1). In the absence of mitogen, similar to the mitogen stimulation, TNF-α, IL-12, and IL-6 productions were induced significantly by all components (Table 2). Cytokine production was decreased at high concentration, whereas the production of IL-12 and IL-6 by exposure to cytoplasm fraction increased in a dose-dependent manner. IFN-γ production was high in cultures with heat-killed cell and cell wall fractions, whereas the production was unaffected with cytoplasm fraction (Table 2). In addition, the production of IL-2 and IL-4 was not detected (data not shown). T helper type 1 (Th1) subset produces IL-2, IFN-γ, and tumor necrosis factor and is vital for cell-mediated immunity. These results indicated that the cellular fractions enhanced the cell-mediated immunity, and that whole cell and cell wall fractions appeared to be more effective than the cytoplasm fraction. IL-12 is also an important cytokine for inducing cellular immunity [14], and this effect may be due to enhanced proliferation, differentiation, or activation of IL-12-secreting cells and Th1 cells. IL-12, TNF-α, and IL-6 are known to be secreted by antigen presenting cells such as macrophages (discussed below), dendritic cells, and/or B cells [25]. The enhanced secretion of these cytokines suggests the possibility that cellular fractions of *Lc. lactis* act on these populations. On the other hand, the cell components enhanced the secretions of IL-2 and IL-4 in the presence of T cell mitogen Con A, implying that they may also stimulate T cells [4].

Table. 2. Effects of heat-killed whole cell, cell wall, and cytoplasm fraction of *Lc. lactis* on the production of cytokines in spleen cells without mitogens stimulation.

μg/ml	Fold control[c]			
	IFN-γ	TNF-α	IL-12	IL-6
WC[a] 10	7.2±1.0*	8.6±1.8*	7.2±0.5*	22±3.2*
50	8.0±0.5*	3.8±0.1*	5.7±0.8*	22±1.7*
100	4.5±0.8*	3.9±0.9*	5.6±0.4*	22±0.8*
500	0.1±0.1	2.8±0.6*	3.4±0.5*	8.0±1.2*
CW[a] 10	6.2±0.6*	3.3±1.3*	3.6±0.1*	18±0.8*
50	7.2±0.6*	-	3.8±0.5*	19±2.8*
100	4.7±0.5*	1.5±0.5	4.1±0.6*	15±4.0*
500	0.2±0.1	5.2±2.1*	3.0±0.4*	6.8±1.3*
CP[a] 10	1.2±0.2	2.3±0.4*	2.3±0.5*	2.6±0.6*
50	1.0±0.2	3.8±0.8*	3.4±0.2*	6.2±0.7*
100	0.2±0	7.1±1.7*	3.4±0.3*	6.0±1.7*
500	-[b]	2.3±0.2*	6.0±0.4*	11±0.7*

[a]WC, whole cell; CW, cell wall; CP, cytoplasm.
[b]-, not detected.
[c]Contents of cytokines were calculated by dividing experimental data by control values (n=3) and are averages of three separate cultures (*$P<0.05$, different from control by Fisher's PLSD).

Effect of *Lc. lactis* Cellular Fraction on the Natural Killer Cell Activity

NK cells can lyse various tumor cells *in vitro* without prior sensitization to tumor antigens [6, 21]. These cells may play an important role in the regulation of tumor development

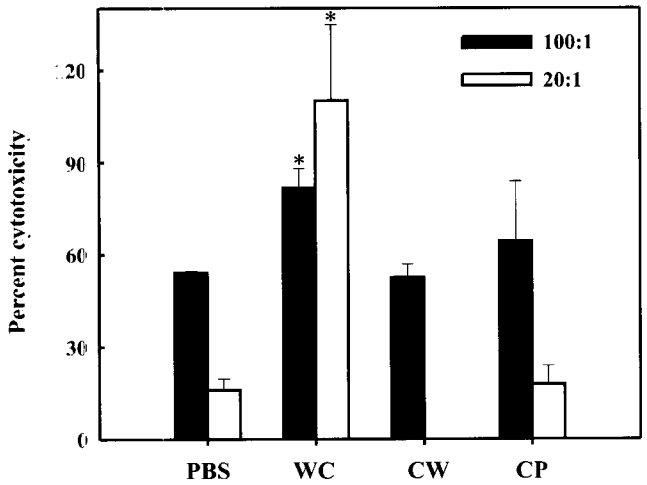

Fig. 1. Percent NK-cell activity against Yac-1 cells in spleen cells stimulated with heat-killed whole cell, cell wall, and cytoplasm fraction of *Lc. lactis* for 5 days at 50 μg/ml; effector cell/target cell ratios were 100:1 and 20:1.
The NK-cell activity shown as cytotoxicity was measured through LDH assay method. The results are expressed as mean±SD. (*$P \leq 0.05$, different from control by Fisher's PLSD) PBS, control; WC, heat-killed whole cell; CW, cell wall; CP, cytoplasm of *Lc. Lactis*.

Table 3. Effects of heat-killed whole cell, cell wall, and cytoplasm fraction of *Lc. lactis* on IL-12 and TNF-α productions in peritoneal exudates cells stimulated with LPS (10 μg/ml).

μg/ml	Fold control[c]	
	TNF-α	IL-12
WC[a] 10	1.7±0.8	0.8±0.4
50	0.9±0.3	0.8±0.6
100	3.8±2.4*	1.1±0.8
500	4.0±3.0*	0.6±0.5
CW[a] 10	3.9±0.4*	1.4±0.5
50	0.8±0.1	1.5±0.3
100	1.1±0.6	0.4±0.4
500	5.1±2.6*	0.1±0.3
CP[a] 10	1.7±0.1	0.6±0.5
50	-[b]	0.3±0.3
100	2.1±0.8	1.8±1.5*
500	2.8±0.6*	1.4±0.8

[a]WC, whole cell; CW, cell wall; CP, cytoplasm.
[b]-, not detected.
[c]Contents of cytokines were calculated by dividing experimental data by control values (n=3) and are averages of three separate cultures (*$P<0.05$, different from control by Fisher's PLSD).

[9, 10, 17, 18] and metastasis [5, 23]. Whether the increase in NK-cell activity reflects an increase in the percentage of NK cells [12] or functional enhancement at a cellular level is not known. Increased NK-cell activities in mice injected with *L. casei* [8], after the oral administration of *L. rhamnosus* [2], have also been reported. The effects of NK-cell activity were determined with spleen cell exposed to the cellular component for 5 days through LDH assay using Yac-1 as target cells. Results revealed NK-cell activity of the spleen cells exposed to heat-killed whole cells was strongly enhanced (Fig. 1). Stimulation with heat-killed whole cell preparation enhanced NK-cell activity significantly; NK cells stimulated with heat-killed whole cell showed strong cytotoxicity at 80–100% against the target cells at effector:target cell ratios of 20:1 and 100:1 (Fig. 1). Thus, as the *in vitro* study revealed, the NK-cell activation by LAB was attributed to its whole cell body, which correlated with its strong effect on IL-12 production.

Effect of *Lc. lactis* Cellular Fraction on the Cytokine Production in Peritoneal Exudates Cells

Macrophages belong to the myeloid lineage and play key roles in inflammation and host defense [13]. Their functions include production of cytokines and mediators, phagocytosis, antigen presentation, and antimicrobial and tumoricidal activities, that all contribute to host defense. In order to assess the activation of macrophage cells, the effects of culture of peritoneal exudates cells with whole cells or their fractions on the production of IL-12 and TNF-α were also determined (Table 3). Although little effect was observed on IL-12 production, TNF-α production was significantly stimulated by heat-killed whole cell and cell wall fractions in peritoneal exudates cells. This may reflect that the stimulation of macrophages is done by cell wall and whole cell.

Attempts have been made to select LAB and their components possessing immunopotentiating activity. [7, 22, 24]. However, the molecular and cellular mechanisms for their immunopotentiation have not yet been well clarified. Although most studies of the immunoactive components of Gram-positive bacteria have focused on cell wall components such as peptidoglycan, other factors may be involved. For example, using sonicated cells of *B. adolescentis*, Hosono *et al.* [7] showed that those cells stimulate Peyer's patch and lymph nodes lymphocytes *in vitro*. Another species of *Bifidobacterium, B. breve* (either whole cell or a cell wall preparation), accelerated *in vitro* proliferation of Peyer's patch cells, particularly B cells [26]. Takahashi *et al.* [22] investigated the interaction of cell fractions of lactic acid bacteria and the immune system, using *B. longum* and *Lb. acidophilus*. Sekine *et al.* [20] demonstrated that cell wall preparations from *B. infantis* induced polymorphonuclear cells and macrophages when injected in the peritoneal cavity. Lee *et al.* [11] also reported that *Bifidobacterium* whole cells and cell-free extracts differentially induced cytokine productions in murine macrophage.

The results presented in this report also suggest that *Lc. lactis* cytoplasm and cell wall fraction as well as whole cell are capable of stimulating lymphocytes and macrophages

to produce significant amounts of several cytokines. Elevated cytokine production, particularly those of IFN-γ and IL-12, correlated with the NK-cell activity.

Further research is needed to determine whether these findings have any relevance *in vivo* or not, and also to identify specific fractions in the cell wall or cytoplasm that have activating properties.

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