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ARTICLE

Effects of *Bifidobacterium* Strains Treated with Gastrointestinal Enzymes on Cytokine Induction in RAW 264.7 Macrophage Cells

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Abstract

The objective of the current study was to compare the abilities of undigested and enzymatically digested bifidobacteria to induce nitric oxide and cytokine release in RAW 264.7 macrophage cells. Nine different *Bifidobacterium strains* derived from herbivorous animals were digested with pepsin and then pancreatin, and the precipitates and supernatants were acquired via centrifugation. The RAW 264.7 cells were incubated with whole cells, the precipitate, or the supernatant, and the macrophage culture supernatants were analyzed with respect to the induction of nitric oxide and cytokines. Pronounced increases in the production of nitric oxide, interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor-α (TNF-α) were observed when cultured with whole cells and the precipitates. It is noteworthy that the precipitates in most of the *Bifidobacterium* strains evidenced a trend toward superior IL-12 release compared with whole cells. The results showed that both whole cells and digested *Bifidobacterium* sp. are effective at stimulating RAW 264.7 cells to induce the production of nitric oxide and cytokines. The pepsin-pancreatin system used in the current study may be useful in unraveling the mechanism by which ingested lactic acid bacteria modulate the induction of macrophage mediators at the cellular level.

Key words: macrophage, nitric oxide, cytokine, bifidobacteria

Introduction

Lactic acid bacteria have been shown to be beneficial to the health and nutrition of consumers by maintaining or improving intestinal microbial balance, reducing serum cholesterol, and augmenting immunopotentiation activity (Fuller, 1991; Gilliland, 1990; Sanders, 1993). It has been reported previously that these probiotic bacteria are capable of enhancing a variety of host immunological functions via activation of macrophages and lymphocytes (Hatcher and Lambrecht, 1993; Kirjavainen *et al*., 1999; Sekine *et al*., 1994), antibody synthesis (Fukushima *et al.,* 1999; Link-Amster *et al*., 1994), T- and B-cell proliferation (Kang *et al.,* 1994; Takahashi *et al.,* 1993), and antitumor activity (Rafter, 1995; Sekine *et al*., 1995). Many prior studies have focused principally on the ability of viable cells, heat-killed cells, peptidoglycan, teichoic acid, and/or cell-free extract to induce several macrophage mediators, including nitric oxide, hydrogen peroxide, and cytokines in macrophages (Amrouche *et al*., 2006; Ouwehand *et al*., 1999). It has been reported previously that immune network systems are modulated through the variety of soluble molecules secreted by immune cells (Arai *et al*., 1990). Activated macrophages are capable of secreting a variety of biologically active substances, such as nitric oxide and interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α). These macrophage mediators perform a pivotal function in a variety of immune responses, including host defense against infection and tumor formation.

However, orally administered live probiotic bacteria are subject to adverse circumstances as they pass through the gastrointestinal tract, including the acidic pH of the stomach, bile acids, and pancreatic juices within the small intestine. This results in poor survival and colonization in the intestinal tract (Clark and Martin, 1994; Marteau *et al*., 1992). The biological functions associated with whole bacterial cells and their cellular components have been extensively evaluated, but few studies of the immune functions of bifidobacteria digested with pepsin followed by pancreatin have been conducted. The principal objective of the current study was to compare the abilities of herbivorous animal–derived *Bifidobacterium* strains, both

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undigested and enzymatically digested, to induce nitric oxide and cytokine release in RAW 264.7 murine macrophage cells.

Materials and Methods

Microorganism and culture conditions

Nine different *Bifidobacterium* strains derived from herbivorous animals were obtained from the Korean Collection for Type Cultures (Dajun, Korea): *Bifidobacterium boum* (KCTC 3227), *Bifidobacterium pseudolongum* (KCTC 3234), *Bifidobacterium cuniculi* (KCTC 3276), *Bifidobacterium thermophilum* (KCTC 3376), *Bifidobacterium animalis* (KCTC 3417), *Bifidobacterium magnum* (KCTC 3422), *Bifidobacterium merycicum* (KCTC 3424), *Bifidobacterium ruminantiym* (KCTC 3425), and *Bifidobacterium adolescentis* (KCTC 3459). All strains were grown anaerobically in MRS broth (Merck, Germany) containing 5% (wt/vol) lactose at 37°C until the late log phase. The cells were then collected via 20 min of centrifugation at 7,000 *g* at 4°C, washed twice in 0.01 M phosphate buffered saline (PBS; pH 7.2), and stored at -20°C until used.

Enzymatic digestion of bifidobacteria

Bacterial cell hydrolysates were prepared via a modified version of the procedure described by Boisen and Fernandez (1995). One gram of whole bacterial cells was suspended in 4 mL of phosphate buffer (0.1 M, pH 6.0), and the pH was adjusted to 2.0 using 1 M HCl. One milliliter of freshly prepared pepsin solution (10 mg/mL in 0.01 M HCl; Merck, Germany) was added to the mixture, followed by 6 h of gentle shaking at 37°C. Following pepsin treatment, 2 mL of phosphate buffer (0.2 M, pH 6.8) was added to the mixture, and the pH was adjusted aseptically to 6.8 using 1 M NaOH. The mixture was then treated further with 1 mL of freshly prepared pancreatin solution (50 mg/mL in 0.2 M phosphate buffer, pH 6.8, Sigma, USA) and shaken for 14 h at 37°C in a water bath. The mixture was heated for 10 min at 72°C in order to inactivate pancreatin. The precipitate (insoluble fraction) and supernatant (soluble fraction) from the mixture were collected via 20 min of centrifugation at 10,000 *g* at 4°C and then lyophilized. For introduction into tissue culture, the whole cell, precipitates, and supernatants were resuspended in Dulbecco's modified Eagles medium to the desired concentration on a dry weight basis and then heated for 30 min at 95°C.

Cell culture

The RAW 264.7 mouse macrophage cell line was acquired from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% (v/v) fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 U/mL) at 37°C in a 5% CO_2 humidified incubator. The cells used in all of the experiments conducted herein were cultured in triplicate at a density of 5×10^5 cells per mL in 24-well tissue culture plates with various concentrations of precipitate or supernatant from hydrolyzed *Bifidobacterium* strains. Lipopolysaccharide (LPS) (*E. coli* O55:B5; Sigma, USA) was used as a positive control. After 48 h, the culture supernatants were analyzed for nitric oxide and cytokines.

Nitric oxide determination

The levels of nitric oxide from the culture supernatants were determined via the Griess reaction (Ding *et al*., 1988). One hundred (100) microliters of culture supernatant was mixed with 2 mL of distilled water, 200 μl of 1% sulfanilamide in HCl, and 200 μL of 0.12% *N*-(1-naphthyl)-ethylenediamine dihydrochloride at room temperature for 10 min; absorbance was measured at 540 nm. The nitrite concentration was then calculated based on a standard curve prepared using sodium nitrite.

Cytokine measurement

TNF-α and IL-6 in the cell culture supernatant were quantitated via enzyme-linked immunosorbent assay (Dong *et al*., 1994). The plates were coated overnight at 4°C with 100 μL of 1 μL/mL rat anti-mouse TNF-α or IL-6 antibodies (Endogen, USA). The plates were then washed three times in 0.01 M PBS (pH 7.4) containing 0.2% (v/v) Tween 20 (PBST). The plates were incubated for 1 h at room temperature with 200 μL of 0.01 M PBS containing 3% (w/v) BSA and then washed three times with PBST. Standard cytokines or samples were added at 50-μL aliquots per well and incubated for 1 h at 37°C. The plates were then washed four times with PBST, incubated for 1 h with 50 μL of 500 ng/mL of biotinylated rat anti-mouse IL-6 or TNF-α antibodies, and washed four times in PBST. Then, 50 mL of streptavidin-horseradish peroxidase conjugate (Pierce, USA) was added to each well, and the plates were incubated for 30 min and washed five times in PBST. Bound peroxidase conjugate was detected via the addition of 100 μL of tetramethylbenzidine and hydrogen peroxide solution (Pierce, USA). The reaction was halted via the addition of 100 μL of 1 M

H_2SO_4, and the absorbance at 450 nm was measured with a Bio-Rad Microplate Reader (Biorad, USA). The cytokine concentration was quantified on the basis of a standard linear dose-response curve. IL-1β and IL-12 concentrations were measured using Opt EIA IL-1β and Opt EIA IL-12p40 (PharMingen, USA) kits, respectively.

Statistical Analysis

All statistical analyses were conducted using the SAS program (2000; SAS Institute, Cary, NC). Significant differences between treatments were tested by analysis of variance, followed by a comparison between treatments using Duncan's multiple range test. The significance level was set at $p<0.05$.

Results and Discussion

The morphology of RAW 264.7 cells

Resident macrophages are capable of synthesizing DNA and proliferating, but activated macrophages lose this ability (Adams and Hamilton, 1987). The morphological changes in RAW 264.7 cells after stimulation with either heat-killed whole cells, or the precipitates or supernatants of digested *Bifidobacterium* strains, are shown in Fig. 1 and are representative of all the strains. The LPS-stimulated cells increased in size, but no such changes were detected in the controls without stimulation (Fig. 1A and 1B). The morphological changes in RAW 264.7 cells incubated with whole cells (Fig. 1C) and with the precipitates (Fig. 1D) were similar to those of LPS, whereas such changes were weak in RAW 264.7 cells incubated with the bacterial supernatant (Fig. 1E). Thus, our findings indicated that the whole cells and precipitates, as well as LPS, influence the activation of RAW 264.7 cells.

Nitric oxide production

RAW 264.7 cells were cultured with whole cells or with the precipitates or supernatants from *Bifidobacterium* strains treated with pepsin and pancreatin, after which the production of nitric oxide in the culture supernatant was measured via the Griess assay (Table 1).

The production of nitric oxide in the RAW 264.7 cells alone was 3.87 μM, whereas it was 25.30 μM in the cells co-stimulated with LPS (1 μg/mL), which is known to be a strong macrophage activator. When RAW 264.7 cells were co-cultured with 10-100 μg/L of either whole cells or precipitates, nitric oxide production generally increased with increases in the concentration of either whole cells or precipitates. It is worth noting that the level of nitric oxide at 10 μg/L of whole cells or precipitates was similar to that of LPS (1 μg/mL). The level of nitric oxide induction appeared to be lower in the macrophages treated with the bacterial supernatants (100 μg/mL). The level of nitric oxide in the bacterial supernatant was higher in strains 3276, 3422, 3424, and 3425 than in the other strains.

Cytokine production by *Bifidobacterium* strains

In an effort to evaluate the effect of digested *Bifidobacterium* strains on the induction of IL-1β, IL-6, IL-12, and TNF-α in the macrophages, RAW 264.7 cells were incubated with either whole cells or the precipitates or supernatants of digested bifidobacteria, and the induction of cytokines in the culture supernatants were subsequently monitored by enzyme-linked immunosorbent assay.

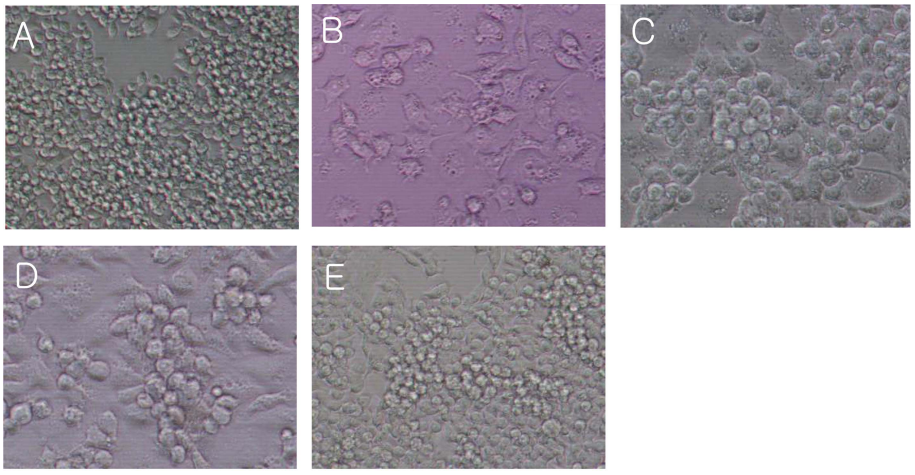

Fig. 1. Morphology of RAW 264.7 cells cultured for 48 h after the addition of Dulbecco's modified Eagle's medium only (A), 1 μg LPS/mL (B), whole cell (100 μg/mL) of *Bifidobacterium merycicum* 3424 (C), precipitate (100 μg/mL) of *Bifidobacterium merycicum* 3424 (D), and supernatant (300 μg/mL) of *Bifidobacterium merycicum* 3424 (E). Magnification A to E: 200×.

Table 1. Effect of bifidobacteria treated with or without gastrointestinal enzymes on nitric oxide production by RAW 264.7 cells

strain	Whole cell		Precipitate		Supernatant	
	10 μg/mL	100	10	100	100	300
B. boum KCTC 3227	28.87^{e}	39.35^{d}	35.09^{b}	51.42^{a}	5.10^{e}	4.86^{e}
B. pseudolongum KCTC 3234	32.67^{de}	54.89^{ab}	38.06^{b}	49.10^{a}	5.72^{de}	7.29^{de}
B. cuniculi KCTC 3276	39.93^{bc}	39.21^{d}	45.15^{a}	55.91^{a}	17.71^{b}	29.49^{b}
B. thermophilum KCTC 3376	42.42^{b}	59.40^{a}	21.14^{d}	50.40^{a}	9.01^{cd}	9.24^{de}
B. animalis KCTC 3417	33.14^{de}	58.91^{a}	40.47^{ab}	51.40^{a}	11.85^{c}	18.93^{c}
B. magnum KCTC 3422	48.58^{a}	60.12^{a}	40.42^{ab}	52.19^{a}	32.45^{a}	33.35^{a}
B. merycicum KCTC 3424	38.10^{bcd}	45.19^{cd}	36.80^{b}	49.98^{a}	20.77^{b}	26.80^{b}
B. ruminantium KCTC 3425	37.98^{bcd}	50.49^{bc}	29.33^{c}	34.78^{b}	20.82^{b}	25.50^{b}
B. adolescentis KCTC 3459	36.26^{cd}	48.19^{bc}	27.79^{c}	53.65^{a}	9.66^{c}	11.05^{d}
Macrophage (-control)	3.87					
LPS 1 μg (+control)	25.30					

The cells (5×10^5/well) were stimulated with or without whole cell and bacterial hydrolysates for 48h in 5% CO_2 incubator. The amount of nitric oxide production in the culture supernatant was measured via the Griess method. The results are expressed as the mean of μM of nitric oxide from the culture supernatant in triplicate.
a,b,c,d,e: Values with different letters are significantly different within the level ($p<0.05$).

Table 2. Effect of bifidobacteria treated with or without gastrointestinal enzymes on IL-1β production by RAW 264.7 cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/mL	100	10	100	100	300
B. boum KCTC 3227	654.85^{de}	1356.33^{c}	885.96^{c}	1527.81^{b}	11.51^{c}	26.33^{ef}
B. pseudolongum KCTC 3234	747.81^{d}	1358.55^{c}	1235.96^{a}	1453.37^{bc}	1.89^{c}	3.00^{f}
B. cuniculi KCTC 3276	712.62^{d}	1439.29^{bc}	1066.33^{b}	1385.59^{cd}	7.44^{c}	244.11^{b}
B. thermophilum KCTC 3376	1227.44^{b}	1521.14^{b}	508.18^{de}	1436.70^{bc}	5.77^{c}	8.56^{ef}
B. animalis KCTC 3417	735.59^{d}	1051.14^{d}	1028.18^{bc}	1184.85^{e}	N.D.	38.92^{e}
B. magnum KCTC 3422	1027.81^{c}	991.51^{d}	1332.26^{a}	1663.00^{a}	177.44^{a}	711.44^{a}
B. merycicum KCTC 3424	211.15^{f}	712.63^{e}	546.33^{de}	1305.96^{d}	43.74^{b}	200.03^{c}
B. ruminantium KCTC 3425	1485.59^{a}	1890.03^{a}	571.52^{d}	1055.96^{f}	21.52^{c}	100.77^{d}
B. adolescentis KCTC 3459	520.03^{e}	1528.18^{b}	413.00^{e}	1518.55^{b}	N.D.	17.81^{ef}
Macrophage (-control)	N.D.					
LPS 1 μg (+control)	585.96					

*N.D.: Not detectable.
Results are expressed as a mean of pg/mL of IL-1β from the cell culture supernatant in triplicate.
a,b,c,d,e,f: Values with different letters are significantly different within the level ($p<0.05$).

The IL-1β level in the RAW 264.7 cells stimulated with LPS (1 μg/mL) was measured at 585 pg/mL and, as had been expected, no IL-1β was detected in the negative-control RAW 264.7 cells alone (Table 2). IL-1β production in the macrophages treated with whole cells or precipitates was markedly induced in all strains. However, the induction of IL-1β in macrophages stimulated with high concentrations of the supernatant (300 μg/mL) was weakly observed in strains 3276, 3422, and 3424.

Bacterial precipitate at a concentration of 10-100 μg/mL evidenced a level of IL-6 induction activity similar to that seen with the whole cells (Table 3). Highly concentrated bacterial precipitate (100 μg/mL) manifested the highest levels of IL-6 induction activity, and this level of activity was similar to that of macrophage induction with LPS (1 μg/mL). It was observed that the induction of macrophages was weak or barely detectable in the bacterial supernatant (100 μg/mL) whereas an increased level of activity was observed in strains 3276, 3422, 3424, and 3425 at higher supernatant concentrations (300 μg/mL). It is worthy of note that IL-12 production in most of the strains was generally higher in the tested range of precipitate concentrations (10-100 μg/mL) than in the bacterial whole cells (Table 4). TNF-α induction activity appeared to occur at a higher level in the macrophages stimulated with whole cells and precipitates at a concentration of 10-100 μg/mL than in macrophages stimulated with LPS, in which it was barely detectable.

Our results generally agree with those of previous studies, i.e., that the production of nitric oxide and several cytokines

Table 3. Effect of bifidobacteria treated with or without gastrointestinal enzymes on IL-6 production by RAW 264.7 cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/mL	100	10	100	100	300
B. boum KCTC 3227	33.49^{d}	95.07^{c}	61.61^{d}	94.90^{b}	N.D.	N.D.
B. pseudolongum KCTC 3234	65.30^{c}	99.23^{b}	80.93^{bc}	92.92^{b}	N.D.	N.D.
B. cuniculi KCTC 3276	32.86^{d}	98.91^{b}	93.61^{a}	99.76^{a}	12.10^{b}	75.72^{b}
B. thermophilum KCTC 3376	83.51^{b}	98.11^{b}	43.36^{f}	100.57^{a}	N.D.	N.D.
B. animalis KCTC 3417	83.68^{b}	99.04^{b}	86.44^{b}	99.55^{a}	N.D.	1.78^{e}
B. magnum KCTC 3422	29.55^{d}	93.55^{c}	97.90^{a}	100.65^{a}	42.81^{a}	89.96^{a}
B. merycicum KCTC 3424	27.08^{d}	84.09^{d}	77.12^{c}	100.16^{a}	8.89^{b}	65.15^{c}
B. ruminantium KCTC 3425	69.76^{c}	102.16^{a}	56.81^{de}	92.13^{b}	6.94^{b}	45.12^{d}
B. adolescentis KCTC 3459	96.02^{a}	102.08^{a}	54.68^{e}	99.78^{a}	N.D.	N.D.
Macrophage (-control)	N.D.					
LPS 1 μg (+control)	95.62					

*N.D.: Not detectable.
Results are expressed as a mean of ng/mL of IL-6 from the cell culture supernatant in triplicate.
a,b,c,d,e,f : Values with different letters are significantly different within the level (p<0.05).

Table 4. Effect of bifidobacteria treated with or without gastrointestinal enzymes on IL-12 production by RAW 264.7 cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/mL	100	10	100	100	300
B. boum KCTC 3227	N.D.	41.14^{d}	71.62^{b}	302.09^{a}	N.D.	N.D.
B. pseudolongum KCTC 3234	24.00^{b}	188.28^{b}	92.09^{a}	272.09^{b}	N.D.	N.D.
B. cuniculi KCTC 3276	N.D.	54.47^{d}	12.09^{e}	97.81^{e}	N.D.	N.D.
B. thermophilum KCTC 3376	42.57^{a}	256.38^{a}	31.14^{cd}	212.57^{c}	N.D.	N.D.
B. animalis KCTC 3417	17.33^{b}	142.57^{c}	N.D.	94.47^{e}	N.D.	N.D.
B. magnum KCTC 3422	14.00^{b}	185.42^{b}	45.43^{c}	210.66^{c}	6.86^{b}	26.85^{c}
B. merycicum KCTC 3424	N.D.	N.D.	N.D.	35.90^{f}	6.81^{b}	25.91^{c}
B. ruminantium KCTC 3425	N.D.	45.43^{d}	N.D.	158.76^{d}	10.66^{b}	39.71^{b}
B. adolescentis KCTC 3459	N.D.	19.43^{e}	19.24^{de}	258.76^{b}	17.81^{a}	48.28^{a}
Macrophage (-control)	N.D.					
LPS 1 μg (+control)	88.76					

*N.D.: Not detectable.
Results are expressed as a mean of pg/mL of IL-12 from the cell culture supernatant in triplicate.
a,b,c,d,e,f: Values with different letters are significantly different within level (p<0.05).

can be increased significantly by the exposure of RAW 264.7 cells to heat-killed whole cells (Park *et al*., 1999; Tejada-Simon and Pestka, 1999). Sakai *et al*. (1996) reported that increased nitric oxide production was observed in J774.1 murine macrophage cells when co-cultured with digested bacterial cell powder derived from *Brevibacterium lactofermentum* treated with lysozymes and proteases. In this study, whole bacterial cells and the precipitates generally evidenced a similar degree of inductive activity on nitric oxide, IL-1β, IL-6, and TNF-α production. Interestingly, the precipitate proved to be a better inducer of IL-12 release than did whole bacterial cells. These findings indicate that cytokine inductive ability in the macrophages could be altered by orally administered bifidobacteria hydrolyzed with gastrointestinal digestive enzymes.

It has been reported that phosphopolysaccharide derived from the cell wall components of gram-positive *Lactobacillus* strains enhanced lymphocyte mitogenicity and macrophage function (Kitazawa *et al*., 1999; Uemura *et al*., 2003). The water-soluble polysaccharide fraction acquired following the sonification of *Bifidobacterium adolescentis* also evidenced immunopotentiation activity (Hosono *et al*, 1997). In particular, the results of the present study indicated that the induction of cytokine activity in RAW 264.7 cells after stimulation with supernatants from several bacterial strains may result from the existence of water-soluble immunopotentiators derived from bifidobacteria as a result of treatment with digestive enzymes, including pepsin and pancreatin. The composition of cell walls of various bacteria may be strain-dependent. Most lactic acid bacteria contain teichoic acid, but their peptidoglycan composition differs (Schleifer and Kandler, 1972). Differ-

Table 5. Effect of bifidobacteria treated with or without gastrointestinal enzymes on TNF-α production by RAW 264.7 cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/mL	100	10	100	100	300
B. boum KCTC 3227	31.76^{c}	52.23^{a}	30.42^{c}	45.23^{bc}	N.D.	0.38^{e}
B. pseudolongum KCTC 3234	54.02^{a}	36.88^{c}	45.26^{b}	41.55^{cd}	0.22^{d}	0.53^{e}
B. cuniculi KCTC 3276	25.14^{d}	46.02^{ab}	27.67^{cd}	41.13^{cd}	2.02^{c}	11.44^{c}
B. thermophilum KCTC 3376	57.73^{a}	17.98^{d}	41.05^{b}	22.26^{f}	0.45^{d}	1.81^{e}
B. animalis KCTC 3417	53.56^{a}	34.65^{c}	40.44^{b}	35.99^{d}	2.15^{bc}	14.71^{b}
B. magnum KCTC 3422	38.83^{b}	40.22^{bc}	53.36^{a}	54.80^{a}	7.45^{a}	17.08^{a}
B. merycicum KCTC 3424	12.54^{e}	40.02^{bc}	21.46^{d}	28.26^{e}	3.31^{b}	9.54^{d}
B. ruminantium KCTC 3425	40.83^{b}	37.01^{c}	30.35^{c}	49.37^{ab}	1.84^{c}	2.01^{e}
B. adolescentis KCTC 3459	12.07^{e}	46.58^{ab}	15.14^{e}	42.16^{c}	1.08^{cd}	10.88^{cd}
Macrophage (-control)	N.D.					
LPS 1 μg (+control)	0.09					

*N.D.: Not detectable
Results are expressed as a mean of ng/mL of TNF-α from the cell culture supernatant in triplicate.
a,b,c,d,e: Values with different letters are significantly different within the level (p<0.05).

ences in the immunopotentiation properties of lactobacilli between strains have been attributed to differences in cell wall structures (Sato *et al*., 1988). The differences in nitric oxide and cytokine induction observed among the *Bifidobacterium* strains in our study may be attributable to variability in their surface structures, which are susceptible to digestive enzymes.

It has been shown that IL-12 can induce a dominant Th1 cell-associated cytokine profile (Trinchieri, 1994). More recently, Kimoto *et al*. (2004) reported that certain *Lactococcus* strains enhance Th1-type immunity. The enhanced IL-12 release observed in the precipitates indicates that the functional properties of the surface structure of whole bacterial cells can be altered by the gut environment, including physical (pH) and chemical (enzyme) conditions, which can affect cell-mediated immunity. When viable *Bifidobacterium lactis* was fed to lactating mice, enhanced local production of anti-β-lactoglobulin IgA was detected in the milk and in the intestines (Fukushima *et al*., 1999). Moreover, both total and anti-β-lactoglobulin IgA levels in the tissue extract of the small intestine wall were significantly higher in mice fed on nonviable *Bifidobacterium longum* (Takahashi *et al*., 1998). IL-6 is critical to mucosal immunity, based on its differentiative effects on IgA-committed B cells and its production in the gut by macrophages, T cells, and other cells (Beagley *et al*., 1989; Vinderola *et al*., 2006). Thus, our results suggest that the enhanced IL-6 production observed with the whole cells or precipitates of *Bifidobacterium* sp. may participate in the stimulation of B cells and thereby result in the induction of IgA production in the gastrointestinal immune system.

The results reported in this study suggest that non-viable bifidobacteria treated with digestive tract enzymes increase the production of nitric oxide and several cytokines and may enhance the intestinal immune functions of the host. It has also been shown that probiotic lactic acid bacteria in feed or food supplements may have immunomodulating properties, even if they are rendered non-viable by the gut environment.

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