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Comparison of Cytokine and Nitric Oxide Induction in Murine Macrophages between Whole Cell and Enzymatically Digested *Bifidobacterium* sp. Obtained from Monogastric Animals

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The principal objective of this study was to compare the effects of whole and hydrolyzed cells (bifidobacteria) treated with gastrointestinal digestive enzymes on the activation of cloned macrophages. Seven different strains of *Bifidobacterium* obtained from swine, chickens, and rats, were digested with pepsin followed by pancreatin and the precipitate (insoluble fraction) and supernatant (soluble fraction) obtained via centrifugation. The RAW 264.7 murine macrophages were incubated with either whole cells, the precipitate, or supernatant at various concentrations. Pronounced increases in the levels of nitric oxide (NO), interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor (TNF)-α were observed in the whole cells and precipitates, but these effects were less profound in the supernatants. The precipitates also evidenced a slight, but significant, inductive activity for NO and all tested cytokines, with the exception of TNF-α in the macrophage model as compared with the whole cells. By way of contrast, TNF-α production when cultured with whole cells (100 ng/ml) resulted in marked increases as compared with what was observed with the precipitates. The results of this study indicated, for the first time, that digested *Bifidobacterium* sp. can induce the production of NO and several cytokines in RAW 264.7 murine macrophage cells. In the current study, it was demonstrated that *Bifidobacterium* strains treated with digestive enzymes, as compared with whole cells, are capable of stimulating the induction of macrophage mediators, which reflects that they may be able to modulate the gastrointestinal immune functions of the host.

Keywords: macrophage, nitric oxide, cytokine, bifidobacteria

The health and nutritional benefits of orally administered probiotic lactic acid bacteria, most notably the lactobacilli and bifidobacteria, has recently begun to garner an increasing amount of attention (Gilliland, 1990; Fuller, 1991).

It has been previously reported that the bifidobacteria can enhance host immunological functions via the activation of macrophages and lymphocytes (Hatcher and Lambrecht, 1993; Sekine *et al.*, 1994; Kirjavainen *et al.*, 1999), antibody synthesis (Link-Amster *et al.*, 1994; Fukushima *et al.*, 1999), and the proliferation of T- and B-cells (Takahashi *et al.*, 1993; Kang *et al.*, 1994). The intake of bifidobacteria has been demonstrated to enhance resistance against infection by pathogenic organisms (Sasaki *et al.*, 1994; Nomoto, 2005), and may also help in the prevention of cancer (Rafter, 1995; Sekine *et al.*, 1995). These immunopotentiating activities have been suggested to be mediated via interactions occurring between immune cells and intact bacterial cells or their components, including peptidoglycan, teichoic acid, and/or cell-free extract (Ouwehand *et al.*, 1999; Amrouche *et al.*, 2006).

Activated macrophages evidence the capacity to induce the production of several cytokines which perform a pivotal function in a variety of immune responses. A variety of studies have demonstrated that viable or heat-killed *Lactobacillus* and *Bifidobacterium* species, as well as certain of their cell components, are capable of stimulating the production of hydrogen peroxide, nitric oxide, and cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α in macrophage cell lines (Miettinen *et al.*, 1996; Park *et al.*, 1999; Tejada-Simon and Pestka, 1999; Cross *et al.*, 2004).

However, the survival and colonization of orally-administered probiotic bacteria in the gastrointestinal environment is generally poor, as these bacteria are susceptible to low acidic pH in the stomach and to bile acid and pancreatic juice within the small intestine (Marteau *et al.*, 1992; Clark and Martin, 1994).

Presently, there is no clear understanding regarding the molecular or cellular basis for immunomodulation by bifidobacteria forced to withstand gastrointestinal conditions. The objective of the current study was to compare the *in vitro* effects of undigested whole cells and enzymatically-digested bifidobacteria on the induction of nitric oxide and IL-1β, IL-6, IL-12, and TNF-α in a RAW 264.7 murine macrophage cell line.

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Materials and Methods

Microorganism and culture condition

Seven different strains of *Bifidobacterium--Bifidobacterium animalis* (KCTC 3126), *Bifidobacterium pseudolongum* (KCTC 3224), *Bifidobacterium thermophilum* (KCTC 3225), *Bifidobacterium cholerium* (KCTC 3275), *Bifidobacterium animalis* (KCTC 3355), *Bifidobacterium pullorum* (KCTC 3370), *Bifidobacterium pseudolongum* (KCTC 3463), all of which were obtained from monogastric animals, were obtained from the Korean Collection for Type Cultures (Daejeon, Korea). All strains were grown anaerobically in MRS broth (Merck, Germany) containing 5% (wt/vol) lactose at 37°C until 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 (pH 7.2) and stored at -20°C until use.

Preparation of antigenic stimulant

Bacterial cell hydrolysates were prepared via a modified version of the procedure described by Boisen and Fernandez (Biosen and Fernandez, 1995). One gram of whole cells was suspended in 4 ml of phosphate buffer (0.1 M, pH 6.0) and adjusted to a pH of 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. After pepsin treatment, 2 ml of phosphate buffer (0.2 M, pH 6.8) was added to the mixture and the pH was aseptically adjusted to pH 6.8 using 1 M NaOH. The mixture was additionally treated 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 then heated for 10 min at 72°C in order to inactivate the pancreatin activity. 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 the preparation of the tissue culture, the precipitates, supernatants, and undigested whole cells were diluted with DMEM to the desired concentration on a dry weight basis, and were then heated for 30 min at 95°C.

Macrophage culture

The RAW 264.7 murine macrophage cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, 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. For the experiments, RAW 264.7 cells were cultured in triplicate at a density of 5×10^5 cells/ml in 24-well tissue culture plates with various concentrations of either whole bacterial cells, precipitates, or supernatants. Lipopolysaccharide (LPS from *E. coli* O55:B5, Sigma) was utilized as a positive control. After 72 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 microliters of culture supernatant was mixed with 2 ml of distilled water, 200 μl of sulfanilamide in HCl and 200 μl of 0.12% N-(1-naphthyl)-ethylenediamine dihydrochloride for 10 min at room temperature and absorbance was measured at 540 nm. Nitrite concentrations were calculated on the basis of a standard curve prepared using sodium nitrite.

Cytokine measurement

TNF-α and IL-6 in the cell culture supernatants were quantified via ELISA (Dong *et al.*, 1994). The plates were coated overnight at 4°C with 100 μl of 1 μg/ml rat anti-mouse TNF-α or IL-6 antibodies (Endogen, USA). The plates were then washed three times with 0.01 M phosphate buffered saline (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 0.01 M PBS containing 3% (w/v) BSA (PBSB), and subsequently washed three times with PBST. Standard cyto-

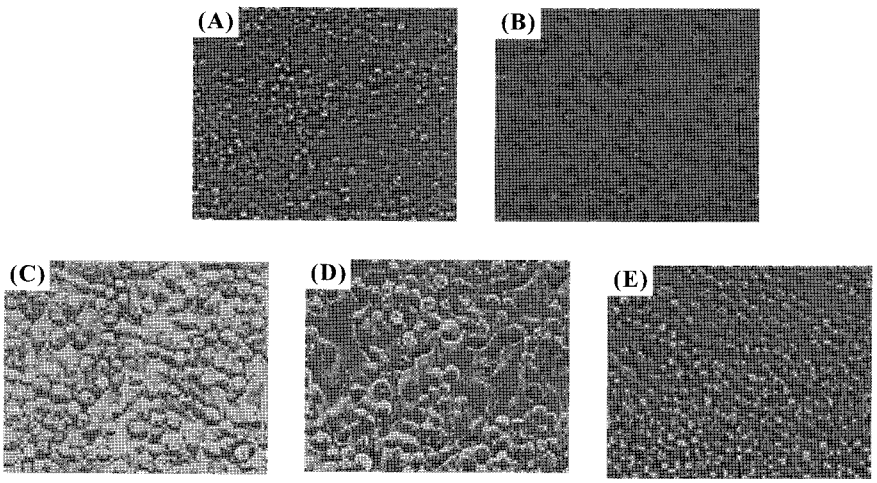

Fig. 1. Morphology of RAW264.7 cells cultured for 72 h after the addition of (A) Dulbecco's modified Eagle's medium only, (B) lipopolysaccharide (1 μg/ml), (C) whole cell (100 μg/ml) of *Bifidobacterium animalis* (3126), (D) precipitate (100 μg/ml) of *Bifidobacterium animalis* (3126), and (E) supernatant (300 μg/ml) of *Bifidobacterium animalis* (3126). Magnification A to E : 200×.

kines or samples were added in 50 μl aliquots per well and incubated for 1 h at 37°C. The plates were washed four times with PBST and incubated for 1 h with 50 μl at a concentration of 500 ng/ml of biotinylated rat anti-mouse IL-6 or TNF-α antibodies and washed four times with PBST. Fifty microliters of streptavidin-horseradish peroxidase conjugate (Pierce, USA) were 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 absorbance was measured at 450 nm using a Bio-Rad Microplate Reader (Biorad, USA). The cytokine concentrations were quantified on the basis of a linear dose-response standard curve. IL-1β and IL-12 were determined using Opt EIA IL-1β and Opt EIA IL-12p40 (PharMingen, USA) kits, respectively.

Statistical analysis

Statistical analyses were conducted using SAS software (SAS, 2000). Significant differences between treatments were assessed via analysis of variance (ANOVA) followed by a comparison between treatments conducted via Duncan's multiple range test, with a 5% level of significance.

Results

The morphology of RAW 264.7 cells

Resident macrophages are able to synthesize DNA and proliferate, but lose this ability when activated (Adams and Hamilton, 1987). Fig. 1 shows the morphological peculiarity of cultured macrophages. When RAW 264.7 cells were cultured alone (Fig. 1A), the majority of cells evidenced circular morphology and normal proliferation, whereas the administration of LPS treatment (Fig. 1B) suppressed proliferation and induced a change in the cellular morphology. When cells

Table 1. Effect of whole cell and bacterial hydrolysates on nitric oxide production by RAW 264.7 murine macrophage cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/ml	100	10	100	100	300
3126	23.60^{b}	30.63^{c}	25.50^{b,c}	45.41^{b,c}	5.91^{c,d}	14.40^{b,c}
3224	25.10^{a,b}	34.95^{a,b,c}	31.15^{b,c}	41.96^{c,d}	5.25^{d}	6.16^{d}
3225	26.43^{a,b}	39.95^{a,b}	24.28^{b,c}	39.75^{c,d}	11.06^{b,c}	12.98^{c,d}
3275	22.25^{b}	29.45^{c}	33.71^{a,b}	54.73^{b}	17.08^{a}	35.41^{a}
3355	21.48^{b}	29.58^{c}	21.83^{c}	32.16^{d}	6.43^{c,d}	7.48^{c,d}
3370	31.78^{a}	40.80^{a}	41.30^{a}	70.18^{a}	13.10^{a,b}	21.00^{b}
3463	27.85^{a,b}	33.43^{b,c}	29.38^{b,c}	39.53^{c,d}	16.30^{a,b}	31.50^{a}
Macrophage (-control)	3.88					
LPS 1 μg/ml (+control)	44.75					

The cells (5×10^5/well) were stimulated with or without whole cell and bacterial hydrolysates for 72 h in 5% CO_2 incubator. The amount of nitric oxide production in the culture supernatant was measured by the Griess method. Results are expressed as a 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 whole cell and bacterial hydrolysates on IL-1β production by RAW 264.7 murine macrophage cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/ml	100	10	100	100	300
3216	278.40^{c}	1056.18^{c,d}	489.88^{c,d}	1281.37^{c}	38.40^{c}	139.51^{d}
3224	332.0^{c}	1310.25^{b,c}	867.29^{b}	1457.66^{c}	N.D.	N.D.
3225	351.37^{c}	1702.11^{a}	295.07^{d}	1740.25^{b}	N.D.	N.D.
3275	254.70^{c}	1336.18^{b,c}	1077.29^{a}	1911.00^{a,b}	81.37^{a,b}	611.37^{b}
3355	594.70^{b}	968.03^{d}	617.66^{b,c}	1723.22^{b}	N.D.	1.37^{e}
3370	392.48^{b,c}	1492.11^{a,b}	1116.92^{a}	2035.07^{a}	63.96^{b}	389.51^{c}
3463	1189.14^{a}	1402.11^{b}	1074.33^{a}	1308.77^{c}	102.48^{a}	763.96^{a}
Macrophage (-control)	N.D.					
LPS 1 μg/ml (+control)	1541.74					

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}Values with different letters are significantly different within the level ($P<0.05$).

Table 3. Effect of whole cell and bacterial hydrolysates on IL-6 production by RAW 264.7 murine macrophage cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/ml	100	10	100	100	300
3126	9.61^{c,d}	51.57^{b}	22.96^{c}	52.71^{b}	N.D.	8.44^{d}
3224	13.45^{c}	52.30^{a,b}	40.18^{b}	51.77^{b}	N.D.	N.D.
3225	6.24^{d}	52.77^{a,b}	12.30^{d}	51.26^{b,c}	N.D.	N.D.
3275	7.44^{d}	51.08^{b}	43.73^{a,b}	53.04^{b}	2.09^{c}	16.09^{c}
3355	32.42^{b}	43.56^{c}	24.10^{c}	49.28^{c}	N.D.	ND
3370	14.06^{c}	55.69^{a}	42.87^{a,b}	56.64^{a}	4.45^{b}	21.98^{b}
3463	42.94^{a}	50.61^{b}	48.25^{a}	51.19^{b,c}	8.39^{a}	36.52^{a}
Macrophage (-control)	N.D.					
LPS 1 μg/ml (+control)	55.05					

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}: Values with different letters are significantly different within the level ($P<0.05$).

Table 4. Effect of whole cell and bacterial hydrolysates on IL-12 production by RAW 264.7 murine macrophage cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/ml	100	10	100	100	300
3216	N.D.	N.D.	2.16^{d}	10.47^{d}	N.D.	N.D.
3224	1.90^{b}	14.28^{a}	7.61^{c}	43.33^{c}	N.D.	N.D.
3225	2.86^{b}	3.81^{c}	8.09^{c}	11.90^{d}	N.D.	7.14^{b}
3275	11.43^{a}	16.66^{a}	16.19^{a}	67.62^{b}	4.28	17.61^{a}
3355	N.D.	2.38^{c}	N.D.	1.90^{e}	N.D.	N.D.
3370	N.D.	8.57^{b}	12.38^{b}	101.90^{a}	N.D.	3.81^{b}
3463	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Macrophage (-control)	N.D.					
LPS 1 μg/ml (+control)	183.33					

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}Values with different letters are significantly different within level ($P<0.05$).

Table 5. Effect of whole cell and bacterial hydrolysates on TNF-α production by RAW 264.7 murine macrophage cells

Strain	Whole cell		Precipitate		Supernatant	
	10 μg/ml	100	10	100	100	300
3216	1.21^{b,c}	22.71^{c}	1.19^{c}	8.49^{b}	N.D.	N.D.
3224	1.07^{b,c}	19.39^{d}	2.96^{a}	6.76^{b,c}	N.D.	N.D.
3225	0.45^{d}	17.30^{d}	0.89^{c,d}	6.94^{b,c}	N.D.	0.22^{c}
3275	0.76^{c,d}	27.55^{a}	1.24^{c}	9.14^{b}	0.34^{a}	3.41^{b}
3355	1.34^{b}	26.33^{a,b}	0.64^{d}	8.01^{b,c}	0.12^{b}	0.18^{c}
3370	0.75^{c,d}	12.24^{e}	1.90^{b}	19.73^{a}	0.36^{a}	6.59^{a}
3463	2.36^{a}	23.66^{b,c}	2.20^{b}	5.91^{c}	0.14^{b}	0.28^{c}
Macrophage (-control)	N.D.					
LPS 1 μg/ml (+control)	4.66					

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 ($P<0.05$).

were co-cultured with heat-killed whole cells (Fig. 1C) or precipitates (Fig. 1D), morphological alterations were clearly observed in association with macrophage activation. This effect was also observed, albeit weakly, in the supernatants (Fig. 1E).

Nitric oxide production

RAW 264.7 cells cultured with either whole cell, the precipitate or supernatant from *Bifidobacterium* strains treated with pepsin and pancreatin, and nitric oxide in the culture supernatants were measured via the Griess assay (Table 1). The untreated RAW 264.7 cells generated 3.88 μM of nitric oxide in the supernatant, whereas the cells co-stimulated with LPS (1 μg/ml), a strong macrophage activator, generated 44.75 μM. When RAW 264.7 cells were co-cultured with 10-100 μg of precipitate per mililiter, nitric oxide production increased with increasing concentrations of precipitate. Interestingly, it was observed that the precipitate induced higher levels of nitric oxide as compared with whole cells, whereas a similar level of nitric oxide was generated in both the precipitate (100 μg/ml) and LPS treatment groups. Nitric oxide induction appeared to be less profound in the macrophages treated with supernatant (100 μg/ml).

Cytokine production by Bifidobacterium strains

In order to evaluate the effects of digested *Bifidobacterium* strains on the induction of IL-1β, IL-6, IL-12, and TNF-α in the RAW 264.7 macrophages were incubated with either whole cell, the precipitates or supernatants of digested bacteria, and cytokine induction in the culture supernatant were subsequently monitored via ELISA.

IL-1β levels in the RAW 264.7 cells stimulated with LPS (1 μg/ml) were evaluated at 1541 pg/ml and, as expected, no IL-1β was detected in the negative control RAW 264.7 cells (Table 2). IL-1β production in the macrophages treated with whole cells or precipitates was markedly induced in all strains. However, IL-1β induction was observed quite weakly in the macrophages stimulated with 100 μg/ml of supernatant.

Bacterial precipitate at low concentrations (10 μg/ml) evidenced higher IL-6 induction as compared to the whole cell, and the activity increased with increasing concentrations (Table 3). Highly concentrated bacterial precipitate (100 μg/ml) evidenced the highest levels of IL-6 induction, and the activity level 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 supernatants (100 μg/ml), whereas a high level of activity was induced at higher supernatant concentrations (300 μg/ml).

RAW264.7 cells co-cultured with LPS (1 μg/ml) generated IL-12 (183 pg/ml) but the level of IL-12 induction was quite weak in the cells stimulated with whole cells or precipitates (10-100 μg/ml). However, strains 3275 and 3370, when treated with precipitates (100 μg/ml), proved able to induce IL-12 production (Table 4).

It should be noted that TNF-α induction activity, in the case of whole cells, was higher than that of the precipitates (Table 5), as it more effectively induces the production of cytokines including IL-1β, IL-6, and IL-12. TNF-α induction activity in the macrophages, even at a supernatant concentration of 300 μg/ml, was quite weak, and was below detectable levels.

Discussion

Immunopotentiating activity has previously been shown to be induced in probiotic lactic acid bacteria that stimulate macrophages to induce a variety of cytokines. The activity of these bacteria has also been associated with the mediation of host defense, inflammatory response, tumoricidal and microbicidal activities. Activation studies have previously been demonstrated to secrete several cytokines via the stimulation of macrophages with live bacteria, heat-killed bacteria, and glutaraldehyde-fixed bacteria (Miettinen *et al.*, 1996; Cross *et al.*, 2004; Kimoto *et al.*, 2004). Moreover, Takahashi (1993, 1998) previously reported that orally administered nonviable *Bifidobacterium longum* could improve intestinal mucosal immunity. According to studies conducted by various researchers, whole cells, peptidoglycan (Tejada-Simon and Pestka, 1999), teichoic acid (Uemura *et al.*, 2003), digested bacterial cells treated with lysozyme and actinase (Sakai *et al.*, 1996), and cell free extract (Hatcher and Lambrecht, 1993) are capable of inducing the production of cytokines, nitric oxide, and hydrogen peroxide. However, previous studies have focused principally on the induction of cytokines from macrophages using heat-killed whole cells or bacterial components. It is important to determine the effects of orally administered probiotic lactic acid bacteria on the gastrointestinal environment at the *in vitro* level. Therefore, we have evaluated the effects on macrophage activation of bifidobateria treated with pepsin at pH 2.0 and pancreatin at pH 7.0. Our results are generally consistent with those of previous studies showing that the production of nitric oxide and several cytokines can be increased significantly by exposing RAW 264.7 cells to heat-killed whole cells (Park *et al.*, 1999). In particular, the precipitates evidenced higher nitric oxide induction and cytokine production activity in the macrophages as compared to whole cells, with the exception of TNF-α. This result indicates that orally administered nonviable probiotic bacteria digested with pepsin and pancreatin could enhance mucosal intestinal immunity. It has also been shown that probiotic lactic acid bacteria in feed or food supplements may evidence immunomodulatory properties, even if they are rendered inviable by the gastrointestinal environment. IL-6 was induced by heat-killed lactobacilli and bifidobacteria in RAW 264.7 murine macrophages (Tejada-Simon and Pestka, 1999). The oral administration of bifidobacteria results in increased production of total IgA or specific IgA in the mouse intestine (Takahashi *et al.*, 1998; Fukushima *et al.*, 1999). In this study, enhanced IL-6 production was observed in RAW 264.7 cells stimulated with whole cells and precipitates. Therefore, our results indicate that *Bifidobacterium* sp. may contribute to the stimulation of B cells, thereby resulting in the induction of Ig A production in the gastrointestinal immune system.

The majority of the tested *Bifidobacterium* strains induced minimal IL-12 production in RAW 264.7 cells. Interestingly, the production of IL-12 as the result of LPS (Gram-negative *E. coli*), which was utilized as a positive control, evidenced enhanced induction activity, whereas Gram-positive

Bifidobacterium resulted in weaker induction activity. This indicates that bifidobacteria has minimal influence on Th1-type immunity. Our results are generally consistent with those of a study that demonstrated markedly increased IL-12 induction in J774.1 cells by *E. coli* as compared with *L. casei Shirota* (Cross *et al.*, 2004). More recently, Kimoto (2004) reported that certain *Lactococcus* strains could enhance Th1 type immunity. It was suggested that non-viable whole cells treated with digestive track enzymes evidence increased nitric oxide production, as well as enhanced production of several cytokines, and are also able to modulate host immune reactions. Also, the *in vitro* pepsin-pancreatin system employed in this study may prove helpful in estimating the efficiency of orally administered probiotic bacteria on digestive tract immunity in hosts. The results reported in this study indicate that probiotic lactic acid bacteria, including non-viable and /or viable bacterial cells treated with gastrointestinal digestive enzymes or bacteria in the digestive tract have the potential to activate the gastrointestinal immune system.

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