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Induction of Cytokines and Nitric Oxide in Murine Macrophages Stimulated with Enzymatically Digested *Lactobacillus* Strains

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Based on observations that lactic acid bacteria have the ability to activate macrophages, we assessed the potential effects of eight different *Lactobacillus* strains treated with gastrointestinal enzymes on the production of nitric oxide and various cytokines in macrophages. RAW 264.7 murine macrophage cells were cultured with either precipitates or supernatants of *Lactobacillus* strains digested with pepsin followed by pancreatin. The increased production of nitric oxide and interleukin (IL)-1β, IL-6, IL-12 and tumour necrosis factor (TNF)-α were observed when cultured with precipitates, and this effect was largely strain-dependent. In contrast, the exposure of RAW 264.7 cells to supernatants produced weaker or nearly undetectable effects in comparison to the effects of exposure to precipitates. The induction of nitric oxide appeared to be unaffected. These results demonstrate that nitric oxide and cytokines were effectively induced when the bacterial precipitate was treated with macrophages. The results of the present study also indicate that *Lactobacillus* strains treated with digestive enzymes are capable of stimulating the production of nitric oxide and cytokines in macrophages, which may modulate the gastrointestinal immune function of the host when it is given as a feed additive.

Keywords: macrophage, nitric oxide, cytokine, lactobacilli

Lactic acid bacteria are widely used as probiotic microorganisms because of the health and nutritional benefits conferred to the host, such as the maintenance of balance in the intestinal microflora, lowering of serum cholesterol, and enhancement of immunopotentiating activity (Gilliland, 1990; Fuller, 1991; Sanders, 1993).

In particular, lactobacilli and bifidobacteria have previously been shown to be related to the enhancement of immune functions, such as 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). These studies were primarily conducted using viable cells, heat-killed cells, peptidoglycan, teichoic acid, and/or cell-free extract.

A number of studies have shown that heat-killed *Lactobacillus* and *Bifidobacterium* species and their cellular components are able to stimulate the production of hydrogen peroxide, nitric oxide and various 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). Lactic acid bacteria in the form of fermented dairy products or feed additives are consumed for their probiotic and health benefits to humans, livestock and pet animals. However, the intestinal settlement of orally administrated probiotic bacteria is poor because they are affected by the low acidic pH of the stomach and by the bile acid and pancreatic juices in the small intestine as they pass through the gastrointestinal tract (Marteau *et al.*, 1992; Clark and Martin, 1994). Since lactobacilli have been suggested to stimulate the immune system, it is of particular interest to study the immunological effect of lactobacilli under gastrointestinal conditions.

The aim of the current study was to evaluate the *in vitro* effect of eight *Lactobacillus* sp. treated with pepsin at an acidic pH and pancreatin on the induction of nitric oxide and IL-1β, IL-6, IL-12 and TNF-α in RAW 264.7 murine macrophage cells.

Materials and Methods

Microorganism and culture condition

Eight different *Lactobacillus* strains, *L. acidophilus* (KCTC 3140), *L. kitasatonis* (KCTC 3155), *L. crispatus* (KCTC 3178), *L. gasseri* (KCTC 3182), *L. murinus* (KCTC 3546), *L. vitulinus* (KCTC 3578), *L. ruminis* (KCTC 3601), and *L. equi* (KCTC 13921) derived from animals were obtained from the Korean Collection for Type Cultures (Daejeon, Korea). All strains were grown in MRS broth (Merck, Germany) at 37°C until the late log phase. Cells were collected by centrifugation at 7,000 rpm for 20 min at 4°C, washed twice with 0.01 M phosphate buffered saline (pH 7.2) and stored at -20°C until used for hydrolysis with digestive enzymes.

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Preparation of antigenic stimulant

Bacterial cell hydrolysates were prepared using a modification of the procedure described by Boisen and Fernandez (1995). One gram of whole bacterial cell 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 gentle shaking at 37°C for 6 h. 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 6.8 using 1 M NaOH. The mixture was further treated with 1 ml of freshly prepared pancreatin solution (50 mg/ml in 0.2 M phosphate buffer; pH 6.8, Sigma) and shaken for 14 h at 37°C in a water bath. The mixture was heated at 72°C for 10 min in order to inactivate the pancreatin. The precipitate (insoluble fraction) and supernatant (soluble fraction) from the mixture were collected by centrifugation at 10,000 rpm for 20 min at 4°C and then lyophilized. The precipitates and supernatants used for tissue culture were diluted with DMEM to the desired concentration on a dry weight basis and then heated at 95°C for 50 min.

Macrophage culture

The mouse macrophage cell line RAW 264.7 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. The cells used for all experiments were cultured in triplicate at a density of 5×10^5 cells/ml in 24-well tissue culture plates with various concentrations of precipitate or supernatant from hydrolyzed *Lactobacillus* strains. LPS (*E. coli* O55:B5, Sigma, USA) was used 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 using the Griess reaction (Ding *et al.*, 1998). One hundred 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, and the absorbance at 540 nm was measured. The nitrite concentration was calculated based on a standard curve prepared using sodium nitrite.

Cytokine measurement

Quantitation of TNF-α and IL-6 in the cell culture supernatant was carried out by ELISA (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 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 of 0.01 M PBS containing 3% (w/v) BSA (PBSB) and then washed three times with PBST. Standard cytokines or samples were added in 50-μl aliquots per well and incubated at 37°C for 1 h. The plates were 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 with PBST. Fifty microliters of streptavidin-horseradish peroxidase conjugate (Pierce, USA) was added to each well, and the plates were then incubated for 30 min and washed five times with PBST. Bound peroxidase conjugate was detected by adding 100 μl of tetramethylbenzidine and hydrogen peroxide solution (Pierce, USA). The reaction was stopped by the addition of 100 μl of 1 M H_2SO_4 and the absorbance at 450 nm was measured using a Bio-Rad Microplate Reader (Biorad). The cytokine concentration was quantified based on a linear dose-response standard curve. The concentrations of IL-1β and IL-12 were determined using Opt EIA IL-1β and Opt EIA IL-12p40 (PharMingen, USA) kits, respectively.

Statistical analysis

All statistical analyses were carried out using the SAS program (SAS, 1996). Significant differences between treatments were tested by analysis of variance (ANOVA), followed by a comparison between treatments using a Duncan's multiple range test, with the level of significance set at $P<0.05$.

Results

Nitric oxide production by Lactobacillus strains digested with enzymes

RAW 264.7 cells were cultured with either the precipitate or supernatant from *Lactobacillus* strains treated with pepsin and pancreatin, and the amount of nitric oxide in the culture supernatant was measured using a Griess assay (Table 1). The production of nitric oxide in the RAW 264.7 cell alone was 3.59 μM, whereas the level of nitric oxide in the supernatant from cells co-stimulated with LPS (1 μg/ml), which is known to be a strong activator of macrophages, was measured at 26.37 μM. When the cells were co-cultured with 10-150 μg of precipitate per ml, the production of nitric oxide generally increased as the concentration of the precipitate increased. It is interesting to note that the level of nitric oxide at precipitate concentrations of more than 50 μg/ml was similar to that of LPS. The induction of nitric oxide in the macrophages treated with supernatant at 10 μg/ml tended to be very low, but tended to gradually increase as the concentration increased. The level of nitric oxide in the bacterial supernatant was lower in strains 3140, 3178, 3601 and 13921 when compared with the other strains.

Cytokine production by digested Lactobacillus strains

To evaluate the effect of digested *Lactobacillus* strains on IL-1β, IL-6, IL-12, and TNF-α in RAW 264.7 macrophages, cells were incubated with either the precipitate or supernatant of digested bacteria, and the induction of cytokines in the culture supernatant was subsequently monitored by ELISA.

The level of IL-1β in RAW 264.7 cells stimulated with LPS (1 μg/ml) was measured at 496 pg/ml and, as expected, no cytokines were detected in the negative control RAW 264.7 cell alone. The production of IL-1β in macrophages treated with precipitate was markedly induced in all strains but strain 3140 (Table 2). However, the induction of IL-1β

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

Strains	Precipitate (μg/ml)			Supernatant (μg/ml)		
	10	50	150	10	50	150
3140	15.97^{d}	$28.83^{b,c,d}$	$31.33^{d,e}$	3.83^{e}	4.70^{e}	$5.29^{e,f}$
3155	$19.08^{c,d}$	$26.57^{c,d}$	21.32^{f}	5.56^{b}	$9.16^{b,c}$	9.68^{d}
3178	10.03^{e}	24.60^{d}	$32.86^{c,d,e}$	5.57^{b}	$5.98^{d,e}$	4.84^{f}
3182	$19.10^{c,d}$	$32.13^{a,b,c}$	27.60^{e}	$5.27^{b,c}$	10.52^{b}	14.05^{c}
3546	22.98^{b}	38.11^{a}	$39.33^{a,b}$	8.43^{a}	16.68^{a}	27.24^{a}
3578	$20.56^{b,c}$	$34.27^{a,b}$	$38.02^{a,b,c}$	$4.60^{b,c}$	10.25^{b}	18.86^{b}
3601	27.22^{a}	$34.38^{a,b}$	42.21^{a}	$3.98^{b,c}$	$7.60^{c,d}$	$7.63^{d,e}$
13921	12.19^{e}	$28.14^{b,c,d}$	$35.10^{b,c,d}$	$4.76^{b,c}$	$7.08^{c,d}$	$6.35^{e,f}$
No bacteria	3.59					
LPS (1 μg/ml)	26.37					

The cells (5×10^{5} per well) were stimulated with or without bacterial hydrolysates for 72 h in a 5% CO_2 incubator.
The amount of nitric oxide production in the supernatant was measured by the Griess method. Results are expressed as the mean production of nitric oxide, in μM, from the culture supernatant in triplicate.
^{a,b,c,d,e,f}Values with different letters are significantly different ($P<0.05$).

Table 2. Effect of bacterial hydrolysates on IL-1β production by RAW 264.7 murine macrophage cells

Strains	Precipitate (μg/ml)			Supernatant (μg/ml)		
	10	50	150	10	50	150
3140	$112.37^{c,d}$	107.18^{d}	208.29^{e}	N.D.	N.D.	N.D.
3155	174.96^{c}	919.03^{b}	$1384.59^{b,c}$	N.D.	N.D.	N.D.
3178	43.85^{d}	488.66^{d}	690.51^{d}	N.D.	N.D.	N.D.
3182	366.44^{b}	$746.44^{b,c}$	1272.00^{c}	N.D.	N.D.	6.81^{c}
3546	292.74^{b}	1143.11^{a}	$1548.66^{a,b}$	N.D.	5.88	123.44^{a}
3578	303.85^{b}	$758.29^{b,c}$	1232.74^{c}	N.D.	N.D.	23.55^{b}
3601	539.03^{a}	1196.07^{a}	$1382.00^{b,c}$	N.D.	N.D.	N.D.
13921	72.37^{d}	$626.07^{c,d}$	1598.29^{a}	N.D.	N.D.	3.11^{c}
No bacteria	N.D.					
LPS (1 μg/ml)	496.81					

N.D.: Not detectable
Results are expressed as the mean production of IL-1β in pg/ml, from the cell culture supernatant in triplicate.
^{a,b,c,d,e}Values with different letters are significantly different ($P<0.05$).

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

Strains	Precipitate (μg/ml)			Supernatant (μg/ml)		
	10	50	150	10	50	150
3140	0.39^{e}	4.42^{e}	24.95^{c}	N.D.	N.D.	N.D.
3155	2.39^{e}	18.03^{d}	57.36^{a}	N.D.	N.D.	N.D.
3178	0.04^{e}	4.66^{e}	48.23^{b}	N.D.	0.02^{b}	0.03^{b}
3182	1.88^{e}	54.12^{b}	$53.21^{a,b}$	N.D.	N.D.	0.11^{b}
3546	19.99^{b}	50.27^{b}	$49.28^{a,b}$	N.D.	0.27^{a}	25.23^{a}
3578	3.6^{e}	67.75^{a}	$56.44^{a,b}$	N.D.	N.D.	0.84^{b}
3601	31.25^{a}	53.29^{b}	$50.24^{a,b}$	N.D.	0.1^{b}	0.14^{b}
13921	0.37	39.07^{c}	$52.03^{a,b}$	0.11	0.07^{b}	0.15^{b}
No bacteria	ND					
LPS (1 μg/ml)	55.18					

N.D.: Not detectable
Results are expressed as the mean production of IL-6, in ng/ml, from the cell culture supernatant in triplicate.
^{a,b,c,d,e}Values with different letters are significantly different ($P<0.05$).

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

Strains	Precipitate (μg/ml)			Supernatant (μg/ml)		
	10	50	150	10	50	150
3140	N.D.	10.46^{c,d}	51.39^{d,e}	N.D.	N.D.	N.D.
3155	N.D.	14.46^{c,d}	255.13^{a}	N.D.	N.D.	N.D.
3178	N.D.	8.12^{c,d}	35.98^{e}	N.D.	N.D.	N.D.
3182	N.D.	4.8^{d}	80.46^{d}	N.D.	N.D.	N.D.
3546	8.46^{b}	76.13^{b}	164.8^{c}	N.D.	N.D.	N.D.
3578	N.D.	18.8^{c,d}	148.13^{c}	N.D.	2.46	27.13
3601	49.13^{a}	96.13^{a}	260.8^{a}	N.D.	N.D.	N.D.
13921	1.933^{c}	21.8^{c}	215.13^{b}	N.D.	N.D.	N.D.
No bacteria	N.D.					
LPS (1 μg/ml)	222.46					

N.D.: Not detectable
Results are expressed as the mean production of IL-12, in pg/ml, from the cell culture supernatant in triplicate.
^{a,b,c,d,e}Values with different letters are significantly different ($P<0.05$).

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

Strains	Precipitate (μg/ml)			Supernatant (μg/ml)		
	10	50	150	10	50	150
3140	33.32^{b}	52.77^{a,b}	62.17^{a}	N.D.	N.D.	N.D.
3155	19.3^{c}	50.62^{a,b}	46.35^{b}	N.D.	N.D.	N.D.
3178	6.78^{e}	25.21^{c}	49.51^{b}	N.D.	N.D.	N.D.
3182	13.48^{c,d}	36.46^{d}	44.61^{b,c}	N.D.	N.D.	N.D.
3546	28.89^{b}	49.43^{b}	44.95^{b,c}	N.D.	N.D.	4.38
3578	17.64^{c,d}	54.11^{a}	46.44^{b}	N.D.	N.D.	N.D.
3601	52.36^{a}	50.84^{a,b}	37.52^{c}	N.D.	N.D.	N.D.
13921	8.28^{e}	42.6^{c}	42.59^{b,c}	N.D.	N.D.	N.D.
No bacteria	N.D.					
LPS (1 μg/ml)	9.22					

N.D.: Not detectable
Results are expressed as the mean production of TNF-α in ng/ml, from the cell culture supernatant in triplicate.
^{a,b,c,d,e}Values with different letters are significantly different ($P<0.05$).

in the macrophages stimulated with 10 or 50 μg/ml of supernatant was not observed, while the induction of IL-1β was induced at 6.8, 123.4, and 23.5 pg/ml in strains 3182, 3546, and 3578, at a concentration of 150 μg/ml, respectively.

IL-6 production in macrophages stimulated with bacterial precipitate from all strains increased with increasing concentrations up to 50 μg/ml (Table 3). The effect of the bacterial supernatant in macrophages inducing IL-6 was very weak and below the level of detection.

The induction of IL-12 in macrophages stimulated with bacterial precipitate from all strains increased in a dose-dependent manner with increasing concentrations of precipitate (Table 4). The production of IL-12 in macrophages stimulated with bacterial supernatant was below the detection limit, with the exception of strain 3578.

The pattern of TNF-α production in the macrophages was similar to that of the other cytokines in that the cells treated with bacterial precipitate induced TNF-α, but the production of TNF-α was not observed in cells stimulated with the bacterial supernatant (Table 5).

Discussion

All vertebrates, including animals and humans, live in symbiosis with a complex population of microorganisms, and this relationship provides the host animal with enhanced resistance to infectious diseases (Fuller, 1991; Fuller and Gibson, 1997). The roles played by lactic acid bacteria and their beneficial effects on the various biological functions of the host have been extensively studied. Early studies demonstrated that the administration of viable lactobacilli activated peritoneal macrophages, thereby increasing their phagocytic capacities (Kato *et al.*, 1983) and stimulated delayed-type hypersensitivity reaction (Bloksma *et al.*, 1979). Lactic acid bacteria have previously been shown to stimulate macrophages, with viable or inactivated bacteria, peptidoglycan, teichoic acid, or cell-free extract exhibiting immunomodulating activities, including the induction of several cytokines

and nitric oxide.

It has been known that the survival of orally administered probiotic microorganisms is poor due to the acidic pH of the stomach as well as the digestive juices secreted by the gastrointestinal tract. Therefore, the artificial treatment of probiotic bacteria digested with pepsin at an acidic pH followed by pancreatin could be used to study the effects of lactic acid bacteria on host immunity. Pepsin is a protease secreted by the stomach, and pancreatin is composed of several enzymes, including protease, amylase, lipase, and ribonuclease. In the present study, Lactobacilli strains treated with a pepsin-pancreatin system were divided into two fractions by centrifugation. One fraction was the precipitate containing insoluble compounds, including partially digested bacteria, and the other was the supernatant, which was comprised of soluble cell components.

From the current study, it is interesting to note that macrophages stimulated with supernatant showed little change in the induction of cytokines and nitric oxide, whereas the induction of the effector molecules was high in macrophages stimulated with the precipitate. Indeed, the results of the present study show that the precipitate plays a critical role in the production of nitric oxide and several cytokines in comparison to the supernatant. The increased production of TNF-α, even at a low precipitate concentration (10 μg/ml), suggests that certain components of the precipitate are particularly effective in the secretion of TNF-α in comparison with the other cytokines tested. It is also interesting to note that, in this study, gastrointestinal enzyme-treated bacteria and their several components have been shown to stimulate the production of nitric oxide and cytokines considerably in RAW 264.7 cells. The observation of pronounced cytokine production by exposure of RAW 264.7 cells to digested *Lactobacillus* sp. is generally in agreement with previous reports that macrophages and lymphocytes are activated in mice following the oral administration of lactobacilli and bifidobacteria (Perdigan *et al.*, 1986; Kirjavainen *et al.*, 1999). The pepsin-pancreatin system employed in the present study may be useful in unravelling the mechanism by which ingested lactic acid bacteria could modulate the induction of macrophage mediators at the cellular level.

Vaccination, infection and inflammation activate a variety of inflammatory cells and induce the activation of a number of oxidant-generating enzymes, such as inducible nitric oxide synthase, which produces nitric oxide from L-arginine (Hibbs, 1991; Hickey, 2001; Bosca *et al.*, 2005). Nitric oxide, a paracrine-acting soluble gas, is known as a pleiotropic biological molecule involved in a variety of physiological and pathological processes, including the regulation of signal transduction, anti-microbial defences (Schoedon *et al.*, 1995; MacMicking *et al.*, 1997), immunomodulation (Gomez-Flores and Weber, 1998), redox regulation (Kim and Ponka, 2002; Forman and Torres, 2001), and apoptosis (Albina and Reichner, 1998). Once produced, nitric oxide has a very short half-life and undergoes spontaneous oxidation to the inactive metabolites nitrite and nitrate (Farzaneh-Far and Moore, 2001). It is generally accepted that the profile of cytokine induction as observed in orally administered *Lactobacillus* strains *in vivo* (Maassen *et al.*, 2000) and the exposure of macrophages to *Bifidobacterium* sp. *in vitro* (Park *et al.*, 1999) were dependent on the dose and bacterial strain used. In the current study, the induction of nitric oxide and cytokines by the precipitate was dose-dependent in most strains. Furthermore, the variety of cytokines produced by the different strains indicates that such activity is strain-dependent.

IL-6 is critical to mucosal immunity based on its effects on the differentiation of 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). The increased production of IL-6 in macrophages stimulated with *Lactobacillus* strains observed in the present study further suggests that this is the key factor for increasing IgA in the feces and Peyer's patches of mice after the oral administration of probiotic lactic acid bacteria (Takahashi *et al.*, 1998; Fukushima *et al.*, 1999). Additionally, the results reported in the present study suggest that even bacterial cells treated with gastrointestinal digestive enzymes are able to stimulate the secretion of several pro-inflammatory cytokines from macrophages and thus could potentially modulate the immune response of the host. Previous studies concerning the immunomodulating activity of probiotic bacteria have largely concentrated on the use of lactic acid bacteria derived from humans and foods, while studies in bacteria from larger domestic animals are less common. Thus, the use of specific strains that are able to produce appropriate levels of cytokines may potentially be used in animal feed as additives for the enhancement of immune responses. Although the results of previous studies and those of the present study favor the use of certain lactobacilli as immunopotentiators, there are still a number of points to be considered. For instance (a) it is critical to know the host cell types that are targeted and stimulated by *Lactobacillus* strains; (b) the cause of the differences observed among the strains in terms of the enhancement of host immunity; (c) since lactic acid bacteria are usually ingested through the daily diet, it is also important to understand the mechanism of action of these bacteria on the immune system; and finally (d) to investigate whether there any potential harmful side effects, especially in pregnant animals or with long periods of exposure?

In conclusion, the results of the present study demonstrate that *Lactobacillus* strains treated with digestive enzymes are capable of stimulating RAW 264.7 macrophage cells to produce nitric oxide and pro-inflammatory cytokines. Moreover, further study is needed to determine whether the in vitro efficacy on the induction of cytokines observed in RAW 264.7 cells would also be observed in animals that ingest these probiotic bacteria.

References

Albina, J.E. and J.S. Reichner 1998. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. *Metastasis Rev.* 17, 39-53.

Beagley, K.W., J.H. Eldridge, F. Lee, H. Kiyono, M.P. Everson, and W.J. Koopman. 1989. Interleukins and IgA synthesis. Human and murine intereukin 6 induce high rate IgA secretion in IgA-committed B cells. *J. Exp. Med.* 169, 2133-2148.

Bloksma, N., E. De Heer, H. Van Dijk, and J.M. Willers. 1979. Adjuvanticity of lactobacilli. I. Differential effects of viable and killed bacteria. *Clin. Exp. Immunol.* 37, 367-375.

Boisen, S. and J.A. Fernandez. 1995. Prediction of the apparent ideal digestibility of protein and amino acids in feedstuffs and feed mixtures for pigs by *in vitro* analyses. *Anim. Feed Sci. Tech.* 5, 29-43.

Bosca, L., M. Zeini, P.G. Traves, and S. Hortelano. 2005. Nitric oxide and cell viability in inflammatory cells: a role for NO in macrophage function and fate. *Toxicol.* 208, 249-258.

Clark, P.A. and J.M. Martin. 1994. Selection of bifidobacteria for use as dietary adjuncts in cultured dairy foods: III-Tolerance to simulated bile concentrations of human small intestines. *Cult. Dairy Prod. J.* 29, 18-21.

Ding, A.H., C.F. Nathan, and D.J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J. Immunol.* 141, 2407-2412.

Dong, W., J.L. Azcona-Olivera, K.H. Brooks, J.E. Linz, and J.J. Pestka. 1994. Elevated gene expression and production of interleukins 2, 4, 5, and 6 during exposure to vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. *Toxicol. Appl. Pharmacol.* 27, 282-290.

Farzaneh-Far, R. and K. Moore. 2001. Nitric oxide and the liver. *Liver* 21, 161-174.

Forman, H.J. and M. Torres. 2001. Redox signalling in macrophages. *Mol. Aspects Med.* 22, 189-216.

Fukushima, Y., Y. Kawata, K. Mizumachi, J. Kurisaki, and T. Mitsuoka. 1999. Effect of bifidobacteria feeding on fecal flora and production of immunoglobulins in lactating mouse. *Int. J. Food Microbiol.* 46, 193-197.

Fuller, R. 1991. Probiotics in human medicine. *Gut.* 32, 439-442.

Fuller, R. and G.R. Gibson. 1997. Modification of the intestinal microflora using probiotics and prebiotics. *Scand. J. Gastroenterol.* 222, 28-31.

Gilliland, S.E. 1990. Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiol. Rev.* 7, 175-188.

Gomez-Flores, R. and R.J. Weber. 1998. Immunomodulation of macrophage functions by opioids. *Adv. Exp. Med. Biol.* 437, 13-19.

Hatcher, G.E. and R.S. Lambrecht. 1993. Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid-producing bacteria. *J. Dairy Sci.* 76, 2485-2492.

Hibbs, J.B. 1991. Synthesis of nitric oxide from L-arginine: a recently discovered pathway induced by cytokines with antitumor and antimicrobial activity. *Res. Immunol.* 142, 565-569.

Hickey, M.J. 2001. Role of inducible nitric oxide synthase in the regulation of leucocyte recruitment. *Clin. Sci.* 100, 1-12.

Kang, K.Y., S.H. Park, and T.B. Choe. 1994. Immunostimulation effect of cell wall components isolated from *Lactobacillus plantarum*. *J. Microbiol. Biotechnol.* 4, 195-199.

Kato, I., T. Yokokura, and M. Mutai. 1983. Macrophage activation by *Lactobacillus casei* in mice. *Microbiol. Immunol.* 27, 611-618.

Kim, S. and P. Ponka. 2002. Nitric oxide-mediated modulation of iron regulatory proteins: implication for cellular iron homeostasis. *Blood Cells Mol. Dis.* 29, 400-410.

Kirjavainen, P.V., H.S. El-Nezami, S.J. Salminen, J.T. Ahokas, and P.F. Wright. 1999. The effect of orally administered viable probiotic and dairy lactobacilli on mouse lymphocyte proliferation. *FEMS Immunol. Med. Microbiol.* 26, 131-135.

Link-Amster, H., F. Rochat, K.Y. Saudan, O. Mignot, and J.M. Aeschlimann. 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol. Med. Microbiol.* 10, 55-64.

Maassen, C.B.M., C. Holten-Neelena, F. Balk, M. Glashouwer, R.J. Leer, J.D. Laman, W.J.A. Boersma, and E. Claassen. 2000. Strain-dependnt induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains. *Vaccine* 18, 2613-2623.

MacMicking, J.D., R.J. North, R. LaCourse, J.S. Mudgett, S.K. Shah, and C.F. Nathan. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc. Natl. Acad. Sci.* 94, 5243-5248.

Marteau, P., P. Pochart, Y. Bouhnik, S. Zidi, I. Goderel, and J.C. Rambaud. 1992. Survival of *Lactobacillus acidophilus* and *Bifidobacterium* sp. in the small intestine following ingestion in fermented milk. A rational basis for the use of probiotics in man. *Gastroenterol. Clin. Biol.* 16, 25-28.

Miettinen, M., J. Voupio-Varkila, and K. Varkila. 1996. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin 10 is induced by lactic acid bacteria. *Infect Immun.* 64, 5403-5405.

Park, S.Y., G.E. Ji, Y.T. Ko, H.K. Hoo, Z. Ustunol, and J.J. Pestka, 1999. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cell exposed to human and commercial isolates of *Bifidobacterium*. *Int. J. Food Microbiol.* 46, 231-241.

Perdigan, G., M.E.N. De Macias, S. Alvarez, G. Oliver, and A.A.P. De Ruiz Holgada. 1986. Effect of perorally administered lactobacilli on macrophage activation in mice. *Infect. Immun.* 53, 404-410.

Sanders, M.E. 1993. Effect of consumption of lactic cultures on human health. *Adv. Food Nutr. Res.* 37, 67-130.

Schoedon, G., M. Schneemann, R. Walter, N. Blau, S. Hofer, and A. Schaffner. 1995. Nitric oxide and infection: another view. *Clin. Infect. Dis.* 21, (Suppl 2): S152-157.

Sekine, K., T. Kasashima, and Y. Hashimoto. 1994. Comparison of the TNF-α level induced by human-derived *Bifidobacterium longum* and rat-derived *Bifidobacterium animalis* in mouse peritoneal cells. *Bifidobact. Microfl.* 13, 79-89.

Statistical analysis systems institute. SAS user's guide: 6.12 edition. 1996 SAS institute inc Cary NC. USA.

Takahashi, T., E. Nakagawa, and T. Nara. 1998. Effects of orally ingested *Bifidobacterium longum* on the mucosal IgA response of mice to dietary antigens. *Biosci. Biotech. Biochem.* 62, 10-15.

Takahashi, T., T. Oka, H. Iwana, T. Kuwata, and Y. Yamamoto. 1993. Immune response of mice to orally administered lactic acid bacteria. *Biosci. Biotech. Biochem.* 57, 1557-1560.

Tejada-Simon, M.V. and J.J. Pestka. 1999. Proinflammatory cytokine and nitric oxide induction in murine macrophages by cell wall and cytoplasmic extracts of lactic acid bacteria. *J. Food Prot.* 62, 1435-1444.

Vinderola, G., G. Perdigon, J. Duarte, E. Farnworth, and C. Mater. 2006. Effects of the oral administration of the products derived from milk fermentation by kefir microflora on immune stimulation. *J. Dairy Res.* 7, 1-8.