Journal of Food Protection, Vol. 62, No. 12, 1999, Pages 1435–1444
Copyright ©, International Association of Milk, Food and Environmental Sanitarians

Proinflammatory Cytokine and Nitric Oxide Induction in Murine Macrophages by Cell Wall and Cytoplasmic Extracts of Lactic Acid Bacteria

MARIA VICTORIA TEJADA-SIMON[1] AND JAMES J. PESTKA[1,2,3]*

[1]*Department of Food Science and Human Nutrition,* [2]*National Center for Food Safety and Toxicology, and* [3]*Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1224, USA*

MS 99-100: Received 9 April 1999/Accepted 19 July 1999

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

ABSTRACT

Cells from a number of bacterial genera have been shown to possess mitogenic and polyclonal activating properties when cultured with cells of the immune system. Based on previously reported health immune-enhancing effects of fermented dairy products, we tested the potentiating effects of representative lactic acid bacteria and their extracts on leukocyte function. Specifically, the effects of in vitro exposure to heat-killed cells of *Bifidobacterium, Lactobacillus acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri,* and *Streptococcus thermophilus,* their cell walls, and their cytoplasmic extracts on proliferation as well as cytokine and nitric oxide (NO) production were examined in the RAW 264.7 macrophage cell line. A similar strategy was applied to murine cultures composed of peritoneal, spleen, and Peyer's patch cells. Both the cell wall and cytoplasmic fractions of lactic acid bacteria were able to stimulate cloned macrophages to produce significant amounts of tumor necrosis factor-α, (interleukin) IL-6, and NO. Pronounced enhancement of IL-6 production by peritoneal cells was observed when cultured with those extracts, whereas, effects were not noted in spleen and Peyer's patch cell cultures from mice. Based on the results, it appears that, as a group, the lactic acid bacteria were capable of stimulating macrophages and possibly other immune cells to produce cytokines and NO, and both their cell walls and cytoplasm contributed to these capacities.

Both cells and products of a large number of bacterial genera have been shown to possess mitogenic and polyclonal activating properties. As mitogens they induce DNA synthesis, blast formation, and ultimately division of leukocytes. One of the most widely studied bacterial products with mitogenic activity is lipopolysaccharide (LPS) that is present in the cell wall of gram-negative bacteria. The basic molecule of LPS consists of a lipid component, lipid A, covalently bound to a heteropolysaccharide of two distinct regions: the core oligosaccharide and the O-specific chain. It can also contain polysaccharide chains of variable lengths. LPS can activate macrophages and induce the release of many mediators and cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 *(29).* LPS has also been shown to exert a mitogenic effect on B cells in mice. Because the structure of LPS is complex and variable even within the same species, the degree of activation can vary. Some bacterial species that are major human pathogens possess a gram-positive cell wall structure. Gram-positive cell walls contain teichoic acid and peptidoglycan. These gram-positive components have also been shown to induce TNF-α and IL-6 production in human monocytes *(10).*

There has been increasing interest in the capacity of certain lactic acid bacteria to function as probiotics when ingested as part of fermented dairy products or as dietary adjuncts. There is an extensive body of literature addressing the possible health benefits associated with the consumption of lactic acid bacteria *(6, 8, 26, 27, 31, 36–39).* 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 stimulation of the immune system. Relative to immunity, Perdigon et al. *(26, 27)* observed enhanced macrophage and lymphocyte activity in mice after administering a mixed culture of *Lactobacillus acidophilus* and *L. casei.* This group also reported activation of peritoneal macrophages in mice after oral administration of *L. casei* and *L. bulgaricus.* Similar results were found for *Streptococcus thermophilus, L. acidophilus* when orally delivered *(28),* and heat-killed *L. casei* administered by injection to mice *(30).* Previous studies conducted in this laboratory found that heat-killed lactic acid bacteria stimulate production of IL-6 and TNF-α in RAW 264.7 macrophage cultures *(18–20).* Thus, a common observation found in these lactic acid bacteria studies is activation of macrophages.

In this study, we assessed the potentiating effects of representative probiotic lactic acid bacteria and their extracts on leukocyte function. We determined: (i) the effects of in vitro exposure to *Bifidobacterium, L. acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri,*

* Author for correspondence. Tel: 517-353-1709; Fax: 517-353-8963; E-mail: pestka@pilot.msu.edu.

TABLE 1. *Cultures used in study*

Bacteria	Strain	Source
Bifidobacterium	Bf-1	Sanofi Bio-Industries
L. acidophilus	La-2	Sanofi Bio-Industries
L. bulgaricus 1489	NCK 23	North Carolina State University
L. casei	ATCC 39539	American Type Culture Collection
L. gasseri ADH	NCK 101	North Carolina State University
L. helveticus	Lr-92	Sanofi Bio-Industries
L. reuteri	ATCC 23272	American Type Culture Collection
S. thermophilus	St-133	Sanofi Bio-Industries

and *S. thermophilus* and their cytoplasmic and cell wall extracts on proliferation, cytokine, and nitric oxide (NO) production by the RAW 264.7 macrophage cell line; and (ii) the same effects in spleen and Peyer's patch lymphocytes as well as in peritoneal cells from mice. The results suggested that lactic acid bacteria as well as their cytoplasmic and cell wall fractions are able to stimulate cloned macrophages to produce large amounts of TNF-α, IL-6, and NO. Although, a pronounced increase in production of IL-6 by peritoneal cells was observed, these effects were not observed in spleen and Peyer's patch cell cultures.

MATERIALS AND METHODS

Lactic acid bacteria fractionation. Representative cultures of *Bifidobacterium, L. acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri, and S. thermophilus* were obtained from three different sources: American Type Culture Collection (Rockville, Md.), Dr. T. R. Klaenhammer (North Carolina State University, Raleigh, N.C.), and Sanofi Bio-Industries (Waukesha, Wis.) (Table 1). From frozen (−80°C) stocks, bacteria were thawed with moderate agitation in a 37°C water bath. Lactobacilli and streptococci were grown in MRS (Difco, Detroit, Mich.) broth *(3)* and bifidobacteria in MRS + 5% (wt/wt) lactose broth at 37°C for 15 h. Cultures (1% vol/vol) were transferred to fresh broth and then incubated at 37°C until exponential phase was reached. The culture was plated after being serially diluted to assess the number of bacteria per ml. Bacteria were recovered by centrifugation at 1,100 × *g* for 15 min.

A schematic representation of the fractionation steps is provided in Figure 1. For heat-killed bacterial fractions, bacteria cultures were quickly chilled to 4°C in an ice/ethanol bath. Bacteria were harvested by centrifugation at 16,000 × *g* for 10 min at 4°C, then washed with sterile distilled water and centrifuged at 16,000 × *g* three times. Bacteria yield and number were determined by wet weight and by plating, respectively. After resuspending in Hank's buffered saline solution (Sigma Chemical Co., St. Louis, Mo.) to achieve a concentration of 25 mg ml^{-1}, the bacteria were aliquoted in 1-ml vials and heated to a temperature of 100°C for 50 min, then quickly chilled to 4°C in an ice bath and stored at −80°C.

For cell wall and cytoplasmic fraction preparations, bacterial cells were harvested and washed as described in the previous section and then subjected to a series of fractionation steps *(10, 36, 40)*. Briefly, after resuspending in sterile distilled water, bacteria were disrupted by sonication for 30 min at 5°C in a Branson Sonifier W-350 (Branson/Sonic Power Co., Danbury, Conn.). The suspension was heated at 60°C for 15 min to inactivate autolytic enzymes. The suspension was centrifuged at 800 × *g* for 30 min at 5°C, and the pellet (unbroken cells) was removed. Cell walls were sedimented from the supernatant by centrifuging at 40,000 × *g* for 30 min. The supernatant was then aliquoted in 1-ml vials and frozen at −80°C (cytoplasmic fraction). The pellet was further treated with protease (20 μg mg^{-1} of crude cell wall), ribonuclease A, and DNAse I (250 μg ml^{-1}) (Sigma) in a 0.1 M Tris-HCl buffer (pH 7.4) at 37°C overnight to eliminate contaminating cytoplasmic material. The cell wall fraction was centrifuged at 800 × *g* for 30 min at 4°C followed by 40,000 × *g* for 30 min, weighed, heated to 90°C for 15 min, aliquoted at a concentration of 25 mg ml^{-1}, and then frozen at −80°C (cell wall fraction). These were filter sterilized through a 0.2-μm filter and kept at −80°C until used for assay.

RAW 264.7 macrophage culture. A murine macrophage cell line obtained from the American Type Culture Collection was used (RAW 264.7 ATCC TIB 71). RAW cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco Laboratories, Chagrin Falls, Ill.), sodium bicarbonate, nonessential amino acids (1 mM) (Gibco), 1 mM sodium pyruvate (Gibco), 10 ml NCTC-135 medium (Gibco), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Sigma). Cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a Neubauer Bright Line counting chamber (American Optical Co., Buffalo, N.Y.). RAW cells were cultured at a final density of 5 × 10^5 cells ml^{-1} in 48-well flat-bottomed tissue culture plates with and without bacterial fractions. *Salmonella* Typhimurium LPS (Sigma; 1 μg ml^{-1}) was used as a positive control for stimulation of RAW cells. Triplicate cultures of this cell line were exposed to 50, 250, or 500 μg (wet weight) of heat-killed bacteria, cytoplasm, or cell wall fraction/ml and incubated for 48 h at 37°C in 7% CO_2. Supernatants were harvested, stored at −80°C for cytokine and NO assay, and cells were used for MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide) assay to determine proliferation/differentiation.

Contamination with exogenous endotoxins was ruled out by using a test with polymyxin B. Various concentrations of polymyxin B (Boehringer Mannheim, Indianapolis, Ind.) (5, 25, 50 μg ml^{-1}) were used as an LPS inhibitor to confirm that fractions were not contaminated with LPS. Bacterial fractions were mixed with the various polymyxin B concentrations, and after a 30-min incubation at room temperature the mixtures were added to the cultures. After 48 h incubation, supernatants were harvested and stored at −80°C for cytokine assay, and cells were used for MTT assay to determine proliferation/differentiation.

Primary cell preparation. Primary cell cultures from B6C3F$_1$ female mice, 8 weeks old (Charles River Labs., Raleigh, N.C.) were used. Mice were sacrificed by cervical dislocation under gentle ether anesthesia. Peritoneal cells were prepared by injecting first 10 ml RPMI-1640 medium containing 10% (vol/vol) heat-inactivated FBS supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 5 × 10^{-5} M 2-mercaptoethanol, 25 mM Hepes buffer, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (10% FBS-RPMI-1640) into the peritoneal cavity of mice with a 10-ml syringe fitted with a 20-gauge needle. The abdomen was massaged, and the liquid was slowly drawn back inside the syringe. The peritoneal fluid was centrifuged at 800 × *g* for 10 min and the supernatant discarded. Ten milliliters of same medium were added to resuspend the cells. Cells were counted with a Neubauer hemacytometer to determine number of cells per ml and cultured to a final density of 2 × 10^5 cells ml^{-1}.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

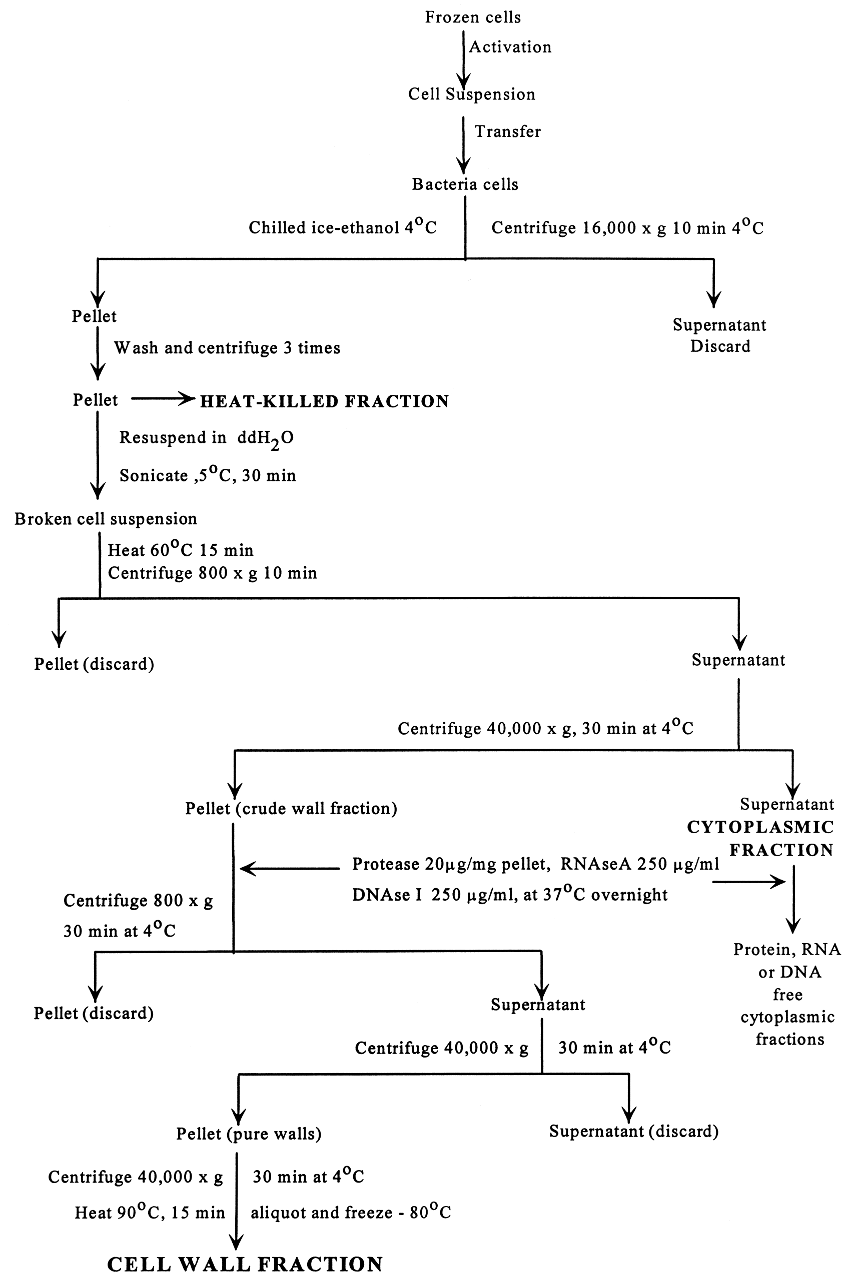

FIGURE 1. *Flow diagram of steps to lactic acid bacteria fractionation.*

Peyer's patches were aseptically removed, teased apart, and passed through an 85-mesh stainless-steel screen. Cells were suspended in 5 ml 10% FBS-RPMI-1640 medium, washed by centrifugation at 450 $\times$ *g* for 10 min, and resuspended in 2 ml of the same medium and counted. Cells were cultured to a final density of 5 $\times$ 10^5 cells ml^{-1}.

Spleens were removed aseptically, teased apart with tissue forceps in 10 ml 10% FBS-RPMI-1640, and centrifuged at 450 $\times$ *g* for 10 min. Erythrocytes were lysed for 5 min at room temperature in 5 ml of a buffer containing 9 parts of 0.16 M ammonium chloride plus 1 part 0.17 M Tris buffer (pH 7.2). Ten milliliters of fresh 10% FBS-RPMI-1640 were added and cells were centrifuged at 450 $\times$ *g* for 10 min, counted, and cultured to a final density of 5 $\times$ 10^5 cells ml^{-1}.

Leukocytes were cultured in a 48-well flat-bottomed tissue culture plate in triplicate with 500 µg (wet weight) of heat-killed cells, cytoplasmic and cell wall fractions of *Bifidobacterium, L. acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri, S. thermophilus* or *Salmonella* Typhimurium LPS 1 µg ml^{-1}. Cells from spleen, Peyer's patches, or peritoneal cavity were incubated at 37°C in 7% CO_2, and supernatants were harvested at time intervals and stored at −80°C for cytokine assay.

Proliferation assay. A colorimetric proliferation assay was performed as described by Mosmann *(22)*. The substrate was prepared fresh weekly and consisted of 5 mg of MTT (Sigma) ml^{-1} in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. This solution was sterilized by passage through a 0.2-µm filter and stored in the dark at 4°C. MTT reagent (150 µl per well) was added to each well after supernatant was removed, and plates were incubated for 75 min in a CO_2 incubator at 37°C. Plates were centrifuged at 450 $\times$ *g* for 10 min, MTT reagent was removed from wells using a syringe with a thin needle to avoid disturbing the pellet, and the crystals formed. Solubilization solution (100% dimethylsulfoxide from Sigma; 450 µl per well) was added to each well and plates were gently agitated to uniformly resuspend the

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

solubilized dye. The solubilized dye (200 μl) was transferred from each well into a 96-well plate and read on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, Calif.) at 570 nm. Unstimulated cell values were used as controls.

Cytokine quantification. Cytokines were quantitated in supernatants by enzyme-linked immunosorbent assay. Immunolon 4 Removawell microtiter strips (Dynatech Laboratories Inc., Chantilly, Va.) were coated overnight at 4°C with 50 μl per well of a purified rat anti-mouse cytokine capture antibody (PharMingen, San Diego, Calif.) in 0.1 M sodium bicarbonate buffer, pH 8.2 (1 μg ml^{-1} [IL-6, TNF-α], 2 μg ml^{-1} [interferon (IFN)-γ]). Plates were washed three times with 0.01 M PBS (pH 7.2) containing 0.2% Tween 20 (PBS-T). Plates were blocked with 300 μl of 3% (wt/vol) bovine serum albumin (BSA) in PBS (BSA-PBS) at 37°C for 30 min and washed 3 times with PBS-T. Standard murine cytokines (PharMingen) or samples were diluted in 10% (vol/vol) FBS-RPMI-1640, and 50-μl aliquots were added to appropriate wells. Plates were incubated at 37°C for 60 min, washed 4 times with PBS-T, and 50 μl of biotinylated rat anti-mouse cytokine detection monoclonal antibody (1.5 μg per well; PharMingen) 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 and 1 additional time with distilled water. Fifty microliters of streptavidin-horseradish peroxidase conjugate (1.5 μg per well; Sigma) diluted in BSA-PBS were added to each well, and plates were incubated at room temperature for 60 min. Plates were then washed eight times with PBS-T and two more times with distilled water, and 100 μl of substrate (10 mM citric phosphate buffer, pH 5.5, containing 0.4 mM tetramethylbenzidine [Fluka Chemical Corp., Ronkonkoma, N.Y.] and 1.2 mM H_2O_2) were added to each well. The reaction was stopped by adding an equal volume (100 μl) of 6 N H_2SO_4. Absorbance was read at 450 nm on a Vmax kinetic microplate reader (Molecular Devices Co.), and cytokine concentrations were quantitated by using Vmax software. The sensitivity of this assay was 0.25 ng ml^{-1} for IL-6, 0.4 ng ml^{-1} for TNF-α, and 1.25 ng ml^{-1} for IFN-γ.

NO determination. Cells were cultured as described in the previous sections for 2 and 5 days, and NO production was assessed by measuring nitrite accumulation, a stable metabolic product of NO, in the culture supernatants *(4).* Nitrite concentrations were determined by the Griess reaction. Equal amounts of *N*-(1-naphthyl)ethylenediamine dihydrochloride (Sigma) (100 mg dissolved in 100 ml of distilled water) and sufanilamide (Sigma) (1 g dissolved in 100 ml of a 5% phosphoric acid solution) solutions were mixed prior to each assay (Griess reagent or chromogenic reagent). Nitrite standards (2 mM stock solution; from 0 to 200 μM) were diluted in the same media in which the cells were suspended. Equal amounts of Griess reagent and $NaNO_2$ standards or samples (100 μl) were placed in a 96-well plate in duplicate and incubated for 5 min at room temperature to allow the chromophore to develop and stabilize. Absorbance was read at 550 nm on the microplate reader, and nitrite concentration was quantitated by using Vmax software. The sensitivity of the assay was 12 μM.

Statistical methods. One-way analysis of variance with Dunnett's test for multiple comparison versus a control group was applied to data, using the Sigma-Stat Analysis System (Jandel Scientific, San Rafael, Calif.). A $P < 0.05$ was considered statistically significant. Results were expressed as relative change in cytokine production compared to control by dividing experimental data by control values (fold control).

RESULTS

Fractionation of bacteria. Lactic acid bacteria yields from MRS broth cultures were between 10 and 15 g $liter^{-1}$. Wall fractions from bacteria cells were prepared from sonicated cells by removal of extraneous material by washing, differential centrifugation, and enzymatic digestion (Fig. 1). The supernatant collected after centrifugation of the sonicated whole cell fraction represented the cytoplasmic content of the bacteria, and the pellet resulted in the crude cell wall. The crude cell wall obtained from the whole cell preparation represented 15 to 30% (wet weight) of the initial bacterial weight, and this is in agreement with the yields generally reported *(40).* Purified cell wall fractions ranged between 36 and 80% (wet weight) with respect to the crude cell wall fraction, depending on the bacterial species.

Effects on proliferation. To assess the effects of lactic acid bacteria fractions and heat-killed cells on macrophage function, RAW 264.7 macrophage cells were stimulated in vitro with 50, 250, and 500 μg (wet weight) ml^{-1} of each bacterial fraction. Cells stimulated with LPS (1 μg ml^{-1}) were used as a positive control, and unstimulated cells were used as a control. The proliferation of macrophage cells, as measured by the MTT assay, was enhanced when RAW cells were cultured for 48 h with 50 μg and 250 μg of any of the fractions. Generally, significant increases with respect to the unstimulated cells (control) were observed at these doses, but this effect diminished when cells were cultured with 500 μg of either heat-killed cells or cytoplasmic fraction (Table 2). LPS significantly depressed proliferation. The results suggested that stimulation with lower concentrations of lactic acid bacteria whole cells or fractions enhanced proliferation of RAW 264.7 cells, but higher concentrations suppressed proliferation.

Effects on cytokine production. Exposure of RAW 264.7 cells to lactic acid bacteria fractions also affected cytokine production in a concentration-dependent manner. For heat-killed cells, production of TNF-α was significantly higher than in control cells with all bacterial fractions demonstrating marked activity (Table 3). In general, TNF-α levels were higher than the levels produced by stimulation with a positive control (LPS, 1 μg ml^{-1}). Cytoplasmic fractions induced marked TNF-α production (Table 3), with significant increases being observed for all concentrations. *L. helveticus, L. reuteri,* and *Bifidobacterium* induced considerable levels of TNF-α at 50 μg and 250 μg, but production decreased dramatically at 500 μg. In contrast, the remaining lactobacilli produced the most TNF-α at the highest concentrations. For cell wall fractions, TNF-α levels significantly increased with concentration in all species tested. Stimulation by cell wall seemed to be more effective than cytoplasmic material and/or LPS relative to TNF-α production, especially for *L. bulgaricus, L. casei,* and *L. helveticus.* In general, these increases were lower than the ones found for heat-killed bacteria.

Stimulation of IL-6 production by the heat-killed lactic acid bacteria and cytoplasmic fractions was also observed (Table 4). However, IL-6 levels were lower than that in-

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

TABLE 2. *Effect of whole cell, cytoplasmic, and cell wall fraction of lactic acid bacteria on proliferation of RAW 264.7 cell line as measured by MTT assay*[a]

	Fold control[b]								
	Whole cell			Cytoplasma			Cell wall		
Bacteria	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	1.41*	1.23*	0.98	1.79*	1.09	0.92*	1.66*	1.68*	1.22*
L. acidophilus La 2	1.35*	1.14*	0.97	1.31*	1.22*	1.05	1.49*	1.65*	1.32*
L. bulgaricus 1489 NCK 231	1.13*	0.88*	0.77*	1.29*	1.14*	0.94	1.23*	1.15*	1.08*
L. casei ATCC 39539	1.26*	1.04	1.03	1.12*	1.09*	0.93*	1.47*	1.16*	0.95
L. gasseri NCK 101	1.25*	1.24*	1.08	1.05	1.15*	1.17*	1.71*	1.40*	1.19
L. helveticus Lr 92	1.22*	1.00	0.91	1.14*	1.05	0.93	1.12*	1.05*	0.91*
L. reuteri ATCC 23272	1.35*	0.95	0.58*	1.36*	1.20*	0.62*	1.49*	1.36*	1.27*
S. thermophilus St-133	1.03	1.08	0.95	1.38*	1.43*	1.25	1.28*	1.56*	1.23*
LPS 1 μg (+control)	0.75*								

[a] RAW 264.7 cells (5×10^5) cells/ml) were cultured with various concentrations of bacteria for 48 h.
[b] MTT relative change was calculated by dividing experimental data by control values ($n = 4$) and are representative of two separate experiments. * $P \leq 0.05$.

duced by the positive control (LPS, 1 μg ml^{-1}). Production of IL-6 by cell wall fractions was also significantly increased for all bacteria species, with *L. bulgaricus* and *L. reuteri* showing the greatest effect. The extent of these increases was, in general, slightly lower than that found for cytoplasmic and heat-killed fractions. Thus, cell walls did not appear as effective at inducing IL-6 as they were inducing TNF-α.

Effects on NO production. The effects of culturing macrophages with cells or fractions on production of NO, another important macrophage mediator, were determined (Table 5). Nitrate levels were used to measure production of NO. In general, NO production either increased or was unaffected as bacterial cell or cell wall fraction concentration was increased, reaching values similar to the ones exhibited by LPS stimulation. Stimulation with the cytoplasmic fraction of lactic acid bacteria had the opposite effect of that observed for heat-killed bacteria and cell wall. NO levels either decreased in a concentration-dependent manner or were unaffected by the cytoplasmic fractions of all bacteria except for *S. thermophilus.*

Polymyxin B neutralization studies. To rule out the possibility that TNF-α, IL-6, and NO production were induced by LPS contamination within the bacteria extracts, RAW 264.7 cells were cultured with up to 50 μg ml^{-1} polymyxin B and with the bacterial fractions or LPS. Polymyxin B is an antibiotic capable of inducing outer membrane permeability in gram-negative bacteria *(23).* The basic molecule of LPS consists of a lipid component (lipid A) covalently bound to a heteropolysaccharide *(34).* Lipid A is responsible for the mitogenic activity attributed to LPS. Polymyxin B is able to inactivate lipid A by attaching to it and affecting overall LPS activity. The results indicated a consistent stimulation effect of bacterial extracts that was independent of the polymyxin B concentration (Fig. 2). In contrast, LPS activity declined when concentration of polymyxin B was increased, affecting proliferation of cells and decreasing production of TNF-α.

TABLE 3. *Effect of whole cell, cytoplasmic, and cell wall fraction of lactic acid bacteria on TNF-α production by RAW 264.7 cells*[a]

	Fold control[b]								
	Whole cell			Cytoplasm			Cell wall		
Bacteria	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	199	336*	395*	100*	89*	3	17*	59*	99*
L. acidophilus La 2	142	739*	816*	100*	125*	169*	41	49*	148*
L. bulgaricus 1489 NCK 231	388*	456*	648*	134*	257*	295*	308*	500*	679*
L. casei ATCC 39539	369*	669*	738*	125*	237*	296*	28	239*	418*
L. gasseri NCK 101	274*	329*	235*	42*	124*	170	34*	77*	110*
L. helveticus Lr 92	549*	756*	1,010*	307*	266*	180*	303*	369*	405*
L. reuteri ATCC 23272	125*	241*	199*	64*	160*	103*	44	149*	228*
S. thermophilus St-133	51*	166*	340*	5	19*	28*	33*	45*	142*
LPS 1 μg (+control)	221*								

[a] RAW 264.7 cells (5×10^5 cells/ml) were cultured with various concentrations of bacteria for 48 h.
[b] TNF-α relative change was calculated by dividing experimental data by control values ($n = 4$) and are representative of two separate experiments. * $P \leq 0.05$.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

TABLE 4. *Effect of whole cell, cytoplasmic, and cell wall fraction of lactic acid bacteria on IL-6 production by RAW 264.7 cells*[a]

	Fold control[b]								
	Whole cell			Cytoplasm			Cell wall		
Bacteria	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	41*	173*	226*	0	65*	49*	0	3	4
L. acidophilus La 2	2	9*	61*	63*	195*	215*	1	2*	5*
L. bulgaricus 1489 NCK 231	37*	58*	83*	22	134*	160*	66*	121*	175*
L. casei ATCC 39539	3	27*	53*	11	79*	211*	1	9*	29*
L. gasseri NCK 101	56*	107*	112*	7	38	110*	0	20*	51*
L. helveticus Lr 92	27*	58*	85*	26*	68*	85*	7	35*	72*
L. reuteri ATCC 23272	61*	112*	195*	31	151*	154*	0	38	116*
S. thermophilus St-133	7*	36*	52*	0	11	6	0	0	3*
LPS 1 μg (+control)	204*								

[a] RAW 264.7 cells (5×10^5 cells/ml) were cultured with various concentrations of bacteria for 48 h.
[b] IL-6 relative change was calculated by dividing experimental data by control values ($n = 4$) and are representative of two separate experiments. * $P \leq 0.05$.

Effects of lactic acid bacteria fractions on primary leukocyte cultures. Peritoneal, spleen, and Peyer's patch cells were incubated with 500 μg of heat-killed, cytoplasmic, or cell wall fractions from lactic acid bacteria as well as with 1 μg LPS for a period of 48 h and analyzed as described in previous sections. Production of TNF-α by peritoneal cells was not detected in any case. Exposure of peritoneal cells to lactic acid bacteria fractions increased IL-6 levels with respect to the control cells (Table 6). Production of IL-6 was significantly higher for all bacteria species, showing very large levels regardless of the cell fraction used. Stimulation with cell wall and cytoplasmic material seemed to be more effective than stimulation with heat-killed bacteria and, in some instances, LPS. *Bifidobacterium, L. bulgaricus, L. casei, L. gasseri, L. helveticus,* and *L. reuteri* appeared to have the greatest effect on these cells. The results suggested that lactic acid bacteria could stimulate peritoneal cells to produce IL-6. Because macrophages are found in peritoneal cells at high concentrations, it is likely that these were the source of the IL-6. However, because peritoneal macrophages were not specifically isolated from other types of cells, we cannot exclude the possibility that some other cells (i.e., T or B cells) besides macrophages were contributing to these high IL-6 levels.

Spleen and Peyer's patch lymphocyte production of IL-6, TNF-α, IFN-γ, or NO were not affected by whole lactic acid bacteria cells or their extracts.

DISCUSSION

Macrophages belong to the myeloid lineage and play a key role in inflammation and in host defense *(20)*. Functions including production of cytokines and mediators, phagocytosis, antigen presentation, antimicrobial, and tumoricidal activity contribute to its role in host defense. These properties are controlled by numerous dynamic stimuli from the tissue microenvironment. The results presented here suggest that lactic acid bacteria and their cytoplasmic and cell wall fractions were capable of stimulating RAW 264.7 macrophages to produce very significant amounts of TNF-α, IL-6, and NO. While these cells and fractions could stimulate IL-6 production in resting peritoneal cells, TNF-α and NO were unaffected. This may reflect that resting

TABLE 5. *Effect of whole cell, cytoplasmic, and cell wall fraction of lactic acid bacteria on NO_3^- production by RAW 264.7 cells*[a]

	Fold control[b]								
	Whole cell			Cytoplasm			Cell wall		
Bacteria	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg	50 μg	250 μg	500 μg
Bifidobacterium Bf-1	11*	12*	15*	5*	6*	2	0	5*	11*
L. acidophilus La 2	7*	18*	22*	17*	12*	7*	0	10*	16*
L. bulgaricus 1489 NCK 231	10*	9*	17*	40*	15*	18*	20*	19*	19*
L. casei ATCC 39539	13*	19*	18*	16*	15*	14*	4*	16*	18*
L. gasseri NCK 101	20*	14*	15*	16*	15*	16*	5*	16*	17*
L. helveticus Lr 92	14*	15*	17*	16*	14*	14*	18*	19*	17*
L. reuteri ATCC 23272	16*	10*	7*	16*	5	0	10*	18*	17*
S. thermophilus St-133	1*	4*	6*	1	5*	8*	0	5*	13*
LPS 1 μg (+control)	15*								

[a] RAW 264.7 cells (5×10^5 cells/ml) were cultured with various concentrations of bacteria for 48 h.
[b] NO_3^- relative change was calculated by dividing experimental data by control values ($n = 4$) and are representative of two separate experiments. * $P \leq 0.05$.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

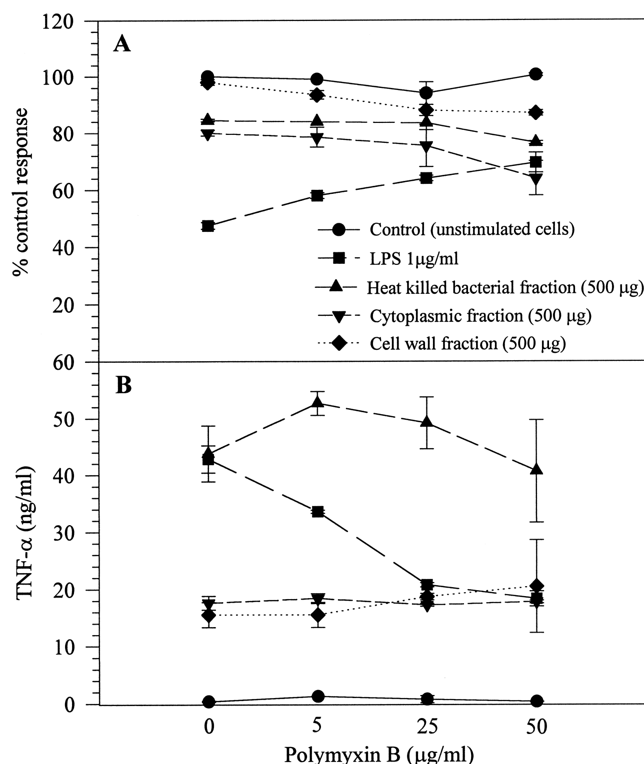

FIGURE 2. *Effect of polymyxin B on* L. casei *ATCC 39539 fractions and LPS stimulation properties. (A) MTT assay; (B) TNF-α production.*

TABLE 6. *Effect of heat-killed, cytoplasmic, and cell wall fractions (500 μg) of lactic acid bacteria on IL-6 production by peritoneal macrophages*[a]

	Fold control[b]		
	Whole cells	Cytoplasm	Cell wall
Bifidobacterium Bf-1	490*	575*	900*
L. acidophilus La 2	588*	650*	430*
L. bulgaricus 1489 NCK 231	400*	700*	400*
L. casei ATCC 39539	780*	1,100*	840*
L. gasseri NCK 101	490*	1,110*	750*
L. helveticus Lr 92	865*	1,265*	910*
L. reuteri ATCC 23272	635*	1,385*	1,000*
S. thermophilus St-133	795*	635*	685*
LPS (1 μg/ml) (+control)	875*		

[a] RAW 264.7 cells (5×10^5 cells/ml) were cultured with 500 μg of bacterial fraction for 48 h.

[b] L-6 relative change was calculated by dividing experimental data by control values (untreated cells) ($n = 4$) and are representative of two separate experiments. * Statistically significant with respect to the control (no stimulation) $P \leq 0.05$.

macrophages were present in our peritoneal cells, whereas the cloned line represents a more highly differentiated macrophage.

The multifunctional cytokines TNF-α and IL-6 are major effector molecules in the response to bacterial agents. TNF-α plays a role in inducing gene expression of several cytokines. TNF-α has been reported to be induced in mouse peritoneal cells within 3 h after the intraperitoneal injection of *Bifidobacterium* cell wall preparation *(33).* TNF-α responses involve increased rates of transcription of particular target genes, often through activation of NF-κB or AP-1 transcription factors. At low concentrations, TNF-α acts as a paracrine and autocrine regulator, making cell surfaces more adhesive. Also, TNF-α stimulates other cell types to produce, cytokines *(13).* At greater quantities, TNF-α enters the blood stream as an endocrine hormone and acts as an endogenous pyrogen. It is able to induce the expression of NO synthase (NOS) that results in conversion of arginine to citrulline and NO. IL-6 is synthesized by macrophages and other cells in response to TNF-α and other signals. IL-6 serves as a growth factor for activated B cells during B-cell differentiation *(1).* It can act as an autocrine factor and also as a cofactor with other cytokines.

Previous research conducted in this laboratory showed that heat-killed lactic acid bacteria stimulated RAW 264.7 cells to produce TNF-α and IL-6 *(18–20).* Our findings are similar to Miettinen at al. *(21),* who reported an induction of the proinflammatory cytokines TNF-α and IL-6 when viable and nonviable lactic acid bacteria were used to stimulate human peripheral blood mononuclear leukocytes. In other studies where RAW 264.7 macrophages were cultured with microbial spores, induction of TNF-α, IL-6 and NO secretion was observed *(11).*

Several investigations have examined the effects of lactic acid bacteria fractions (especially *Bifidobacterium* strains) on immunopotentiating activity. For example, using sonicated cells of *B. adolescentis,* Lee et al. *(16)* showed that those cells stimulate Peyer's patch and lymph nodes lymphocytes in vitro. Another species of *Bifidobacterium, B. breve,* accelerated in vitro proliferation of Peyer's patch cells, particularly B cells *(41).* The proliferation of B cells is enhanced when the supernatant of plastic adherent cells cultured with *B. breve* (either whole cell or a cell wall preparation) is added. This indicates that *B. breve* activates plastic-adherent cells and that these cells secrete a soluble factor that enhances proliferation of B cells. Takahashi et al. *(36)* investigated the interaction of cell fractions of lactic acid bacteria with the immune system, using *B. longum* and *L. acidophilus.* In mice fed *B. longum* for more than 8 weeks, a strong antibody response was detected to the cytoplasmic fraction but not the cell wall fraction. In mice fed *L. acidophilus* for more than 6 weeks, antibody responses were detected against the cytoplasmic and cell wall fractions. Sekine et al. *(32)* demonstrated that cell wall preparations from *B. infantis* induced polymorphonuclear cells and macrophages when injected in peritoneal cavity. These cells inhibited the growth of tumor cells in vitro.

During fractionation procedures of bacterial cells that contain large amounts of lipids, disintegration can be impaired by clumping, and for this reason they are sometimes subjected to preliminary solvent extraction at room temperature *(40).* However, such procedures have the disadvantages that there is considerable destruction of sugars and the presence of organic solvent residues, that is not desirable due to the possibility of inadvertent effects in cell cultures. Thus, less drastic methods of wall isolation from cells

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

were used in this study, such that polymers (peptidoglycan), mucopeptides, teichoic acids, polysaccharides, and other components may be simultaneously present.

The heat-killed, cytoplasmic, and cell wall concentrations required in this study to elicit maximal cytokine production were substantially higher than the concentrations of LPS (1 μg ml^{-1}) that induce comparable levels of TNF-α and IL-6. Some components of pathogenic gram-positive bacteria (streptococci, staphylococci, and other genera) produce significant amounts of TNF-α and IL-6 at concentrations above 100 ng to 1 μg of cell wall ml^{-1} with maximal production requiring 10 to 100 μg of cell wall material ml^{-1} in human monocytes *(10).* Thus, our results for cell walls were consistent with that found for other gram-positive microbes.

In spite of their different chemical nature, gram-positive bacterial extracts and LPS appear to induce similar biological effects and thus might activate the same mediator system. Relatedly, peptidoglycan present in gram-positive bacteria has been reported to induce inflammation *(34).* Peptidoglycan also stimulates release of numerous macrophage products including endogenous cytokines, intermediates, and prostaglandins *(1).* Their induced secretion might well have important implications with respect to the inflammatory process that occurs after exposure to peptidoglycan *(5).* The cell walls of other nonlactic gram-positive bacteria at a dose of 10 μg (1×10^6 cells) produce, in vitro, maximum activation of the peritoneal macrophages of guinea pigs due to the presence of both peptidoglycan and peptidoglycolipid *(35).* Isolated peptidoglycan-polysaccharide polymer fractions from cell walls have been shown also to produce these effects *(24).* Recently, De Ambrosini et al. *(2)* found that specific strains of *L. casei, L. acidophilus,* and *Lactococcus lactis* contained glycerol teichoic acids and A4a-type murein in their peptidoglycan. When they tried to induce phagocytosis in peritoneal macrophages with these fractions, only the *L. casei* peptidoglycan exhibited activity. Proteins and teichoic acids stimulate macrophage cells to release cytokines and NO *(7).* Loss of an acyl group or any other modification can, in some cases, abrogate cytokine stimulating activity *(9).*

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, water-soluble polysaccharides can be immunostimulatory as has been described in bifidobacteria *(12).* Notably, b-glucans activate macrophages, natural killer cells, and killer T cells *(25)* with their action being related to molecular weight, degree of branching, and conformation. Incubation of peritoneal macrophages with b-glucans in the presence of TNF-α or IL-6 augments NO synthesis. It has also been demonstrated that some types of glycolipids from gram-positive bacteria activate human monocyte IL-6 expression by an identical pathway to LPS *(42).* Those glycolipids were, however, unable to induce TNF-α release.

NO production was included in this study based on a metabolic pathway existing in the macrophage and other cells that converts L-arginine to reactive nitrogen intermediates, via an oxidative enzymatic pathway involving an NOS *(4).* NO produced in vivo reacts with molecular oxygen to generate other reactive oxides of nitrogen, such as dinitrogentrioxide (N_2O_3) and dinitrogentetroxide (N_2O_4). These can react with water to form nitrite that is further oxidized to nitrate in vivo. It is known that NO is an important mediator in the nonspecific host defense against microbes and tumors. NO is one of the cytotoxic agents by which activated macrophages can kill bacteria, tumor cells, and a variety of other pathogens but also normal tissue cells during autoimmune reactions *(15, 17).* Chemically, NO is a small lipophilic molecule, and only a few microorganisms can block its entry. Cytokines and microbial products can synergistically stimulate inducible NOS expression. In mouse peritoneal macrophages, IFN-γ seems to be responsible for this stimulation *(14).* In RAW 264.7, LPS also stimulates iNOS expression. Ingestion of microorganisms generally elicits TNF-α production and this cytokine exerts an autocrine stimulus increasing the antimicrobial action of the macrophage, in particular by inducing the production of NO.

Taken together, these data strongly suggest that both the cell wall and cytoplasmic fractions of lactic acid bacteria can stimulate macrophages to produce cytokines. Elevated cytokine production, especially that of IL-6 and TNF-α, were consistent with later increased NO synthesis. Further purification of the lactic acid bacteria fractions produced here may reveal the identity of one or more stimulatory components. If activation by these bacterial fractions plays any role in vivo, one key factor that could influence this effect might be the requirement for prolonged exposure. It was reported that bacterial cell wall components can persist in tissues for long periods of time *(5).* Thus, the cell wall may be the most likely candidate to provide a continuous stimulus required for activation. Under normal conditions, these cell wall components may come in contact with leukocytes found in the intestine or the peritoneum. Leukocytes could be activated either directly by bacterial products as well as by cytokines secreted by peritoneal macrophages or possibly other immune cells. Inflammatory mediators, such as TNF-α, IL-6, act on the neighboring cells and quickly amplify the cellular response. Following diffusion in the tissue, these cytokines may activate local macrophages, fibroblasts, and endothelial cells that also produce mediators and thus contribute to the overall immune response. Further research is needed to determine if these findings have relevance in vivo and to identify the specific fractions in the cell wall and cytoplasm of lactic acid bacteria that have activating properties.

ACKNOWLEDGMENTS

This work was supported by the U.S. Department of Agriculture National Research Initiative Competitive Grants Program, the National Dairy Council, the Michigan State University Crop and Food Bioprocessing Center, and the Michigan State Agricultural Experiment Station. M. V. Tejada-Simon was a recipient of a doctoral fellowship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Ministerio de Agricultura, Pesca y Alimentación, Spain).

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

REFERENCES

1. Abbas, A. K., A. H. Lichtman, and J. S. Pober. 1994. Cellular and molecular immunology, 2nd ed. Saunders Co., Philadelphia, Pa.
2. De Ambrosini, V. M., S. Gonzalez, G. Perdigon, A. P. de Ruiz Holgado, and G. Oliver. 1996. Chemical composition of the cell wall of lactic acid bacteria and related species. Chem. Pharm. Bull. 44(12): 2263–2267.
3. De Man, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of *Lactobacilli.* J. Appl. Bacteriol. 23(1):130–135.
4. Dietert, R. R., J. H. Hotchkiss, R. E. Austic, and Y. J. Sung. 1995. Production of reactive nitrogen intermediates by macrophages, chapter 8. *In* R. R. Dieter, J. H. Hotchkiss, R. E. Austic, and Y. J. Sung (ed.), Methods in immunotoxicology, 2. Wiley-Liss, New York.
5. Dziarski, R. 1982. Studies on the mechanism of peptidoglycan- and lipopolysaccharide-induced polyclonal activation. Infect. Immun. 35(2):507–514.
6. Gilliland, S. E. 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. 87:175–188.
7. Gomez-Flores, R., C. Rodriguez-Padilla, R. T. Mehta, L. Galan-Wong, E. Mendoza-Gamboa, and R. Tamez-Guerra. 1997. Nitric oxide and TNF-α production by murine peritoneal macrophages activated with a novel 20 kDa protein isolated from *Bacillus thuringensis* var. *thuringensis* parasporal bodies. J. Immunol. 158:3796–3799.
8. Halpern, G. M., G. Vruwing, J. Van de Water, C. L. Keen, and M. E. Gershwin. 1991. Influence of long-term yoghurt consumption in young adults. Int. J. Immunother. VII:205–210.
9. Henderson, B., and M. Wilson. 1996. Cytokine induction by bacteria: beyond lipopolysaccharide. Cytokine 8(4):269–282.
10. Heumann, D., C. Barras, A. Severin, M. P. Glauser, and A. Tomasz. 1994. Gram positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin 6 by human monocytes. Infect. Immun. 62(7):2715–2721.
11. Hirvonen, M. R., A. Nevalainen, N. Makkonen, J. Monkkonen, and K. Savolainen. 1997. *Streptomyces* spores from moldy houses induce nitric oxide, TNF-α and IL-6 secretion from RAW 264.7 macrophage cell line without causing subsequent cell death. Environ. Toxicol. Pharmacol. 3:57–63.
12. Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S. Kaminogawa. 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101–4. Biosci. Biotech. Biochem. 61(2): 312–316.
13. Janeway, C. J., and P. Travers (ed.). 1997. Immunobiology: the immune system in health and disease, 3rd ed. Garland Publishing Inc., New York.
14. Kim, Y. M., B. S. Lee, K. Y. Yi, and S. G. Paik. 1997. Upstream NF-kB site is required for the maximal expression of mouse inducible nitric oxide synthase gene in interferon-γ plus lipopolysaccharide-induced RAW 264.7 macrophages. Biochem. Biophy. Res. Commun. 236:655–660.
15. Laskin, J. D., S. E. Heck, and D. L. Laskin. 1994. Multifunctional role of nitric oxide in inflammation. Trends Endocrinol. Metab. 5: 377-382.
16. Lee, J. H., A. Ametani, A. Enomoto, Y. Sato, H. Motoshima, and F. A. K. J. Ike. 1993. Screening for the immunopotentiating activity of food microorganisms and enhancement of the immune response by *Bifidobacterium adolescentis* M101-4. Biosci. Biotech. Biochem. 57(12):2127–2132.
17. MacMicking, J., Q. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol. 15:323–350.
18. Marin, M. L., J. H. Lee, J. Murtha, Z. Ustunol, and J. J. Pestka. 1997. Differential cytokine production in clonal macrophage and T-cell lines cultured with bifidobacteria. J. Dairy Sci. 80:2713–2720.
19. Marin, M. L., M. V. Tejada-Simon, J. H. Lee, J. Murtha, Z. Ustunol, and J. J. Pestka. 1998. Stimulation of cytokine production in clonal macrophage and T cell models by *Streptococcus thermophilus*: comparison with *Bifidobacterium* spp. and *Lactobacillus bulgaricus.* J. Food Prot. 61(7):859–864.
20. Marin, M. L., M. V. Tejada-Simon, J. Lee, Z. Ustunol, and J. J. Pestka. 1997. Effects of *Lactobacillus* spp. on cytokine production by RAW 264.7 macrophages and EL-4 thymoma cell lines. J. Food Prot. 60(11):1364–1370.
21. Miettinen, M., J. Vuopio-Varkilla, 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(12):5403–5405.
22. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65(1–2):55–63.
23. Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49(1):1–32.
24. Novakovic, S., and I. Boldogh. 1994. In vitro TNF-α production and in vivo alteration of TNF-α RNA in mouse peritoneal macrophages after treatment with different bacterial derived agents. Cancer Lett. 81:99–109.
25. Ohno, N., T. Hashimoto, Y. Adachi, and T. Yadomae. 1996. Conformation dependency of nitric oxide synthesis of murine peritoneal macrophages by b-glucans in vitro. Immunol. Lett. 52:1–7.
26. Perdigon, G., M. E. Nader de Macias, S. Alvarez, M. Medici, G. Oliver, and A. Pesce de Ruiz Holgado. 1986. Effect of a mixture of *Lactobacillus casei* and *Lactobacillus acidophilus* administered orally on the immune system in mice. J. Food Prot. 49(12):986–989.
27. Perdigon, G., M. E. Nader de Macias, S. Alvarez, G. Oliver, and A. A. Pesce de Ruiz Holgado. 1986. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect. Immun. 53(2): 404–410.
28. Perdigon, G., M. E. Nader de Macias, S. Alvarez, G. Oliver, and A. A. Pesce de Ruiz Holgado. 1987. Enhancement of immune response in mice fed with *Streptococcus thermophilus* and *Lactobacillus acidophilus.* J. Dairy Sci. 70:919–926.
29. Roth, R. A., J. R. Harkema, J. J. Pestka, and P. E. Ganey. 1997. Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? Toxicol. Appl. Pharmacol. 147: 300–311.
30. Saito, H., H. Tomioka, and K. Nagashima. 1987. Protective and therapeutic efficacy of *Lactobacillus casei* against experimental murine infections due to *Mycobacterium fortuitum* complex. J. Gen. Microbiol. 133:2843–2851.
31. Salminen, S., and A. von Wright. 1993. Lactic acid bacteria. Marcel Dekker, New York.
32. Sekine, K., T. Kawashima, and Y. Hashimoto. 1994. Comparison of the TNF-α levels induced by human-derived *Bifidobacterium longum* and rat-derived *Bifidobacterium animalis* in mouse peritoneal cells. Bifidobacteria Microflora 13(2):79–89.
33. Sekine, K., E. Watanabe-Sekine, T. Toida, T. Kasashima, T. Kataoka, and Y. Hashimoto. 1995. Adjuvant activity of the cell wall of *Bifidobacterium infantis* for in vivo immune responses in mice. Immunopharmacol. Immunotoxicol. 16(4):589–609.
34. Stewart-Tull, D. E. S., and M. Davies. 1985. Immunology of peptidoglycan-polysaccharide polymers from cell walls of group A streptococci, p. 91–118. *In* D. E. S. Stewart-Tull and M. Davies (ed.), Immunology of the bacterial cell envelope. John Wiley and Sons, New York.
35. Takada, H., M. Tsuimoto, S. Kotani, S. Kusumoto, M. Inage, T. Shiba, S. Nagao, I. Yano, S. Kawata, and K. Yokogawa. 1979. Mitogenic effect of bacterial cell walls, their fragments and related synthetic compounds on thymocytes and splenocytes of guinea-pigs. Infect. Immun. 25:645–652.
36. 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(9):1557–1560.
37. Tejada-Simon, M. V., J. H. Lee, Z. Ustunol, and J. J. Pestka. 1999. Ingestion of yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium* spp. to potentiate immunoglobulin A responses to cholera toxin in mice. J. Dairy Sci. 82:649–660.
38. Tejada-Simon, M. V., Z. Ustunol, and J. J. Pestka. 1999. Effects of lactic acid bacteria on basal cytokine mRNA and immunoglobulin levels in the mouse. J. Food Prot. 62(3):287–291.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021

39. Tejada-Simon, M. V., Z. Ustunol, and J. J. Pestka. 1999. Ex vivo effects of lactobacilli, streptococci and bifidobacteria ingestion on cytokine and nitric oxide production in a murine model. J. Food Prot. 62(2):162–169.
40. Work, E. 1971. Cell walls, chapter XIII. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology. Academic Press, New York.
41. Yasui, H., and M. Ohwaki. 1991. Enhancement of immune response in Peyer's patch cells cultured with *Bifidobacterium breve.* J. Dairy Sci. 74:1187–1195.
42. Zhang, Y., M. Broser, and W. M. Rom. 1994. Activation of the interleukin 6 gene by *Mycobacterium tuberculosis* and lipopolysaccharide is mediated by nuclear factors NF-IL-6 and NF-kB. Proc. Natl. Acad. Sci. USA 91:2225–2229.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/62/12/1435/1663805/0362-028x-62_12_1435.pdf by guest on 28 October 2021