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Ex Vivo Effects of Lactobacilli, Streptococci, and Bifidobacteria Ingestion on Cytokine and Nitric Oxide Production in a Murine Model

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ABSTRACT

Increasing numbers of functional foods and pharmaceutical preparations are being promoted with health claims based on the potential probiotic characteristics of lactic acid bacteria and on their capacity for stimulating the host immune system. However, the specific immune effects of oral administration of these microbes still remains undefined. In this study, we tested the hypothesis that production of immunologic mediators by leukocytes in mice is affected by orally administered lactic acid bacteria. The specific objectives of this study were to evaluate the effects of exposure to eight different lactic acid bacteria in mice on ex vivo cytokine and nitric oxide production in leukocyte cultures. Mice were gavaged with 1×10^9 viable bacteria and peritoneal, Peyer's patch and splenic leukocytes were isolated 8 h later. These were cultured for 2 or 5 days in the presence or absence of mitogens and then interleukin (IL)-6, IL-12, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and nitric oxide production was measured. The results revealed that *Lactobacillus acidophilus* and *L. casei* potentiated IL-6 and IL-12 production by peritoneal cells whereas *L. acidophilus* upregulated IFN-γ and nitric oxide. In contrast, *L. helveticus, L. gasseri, L. reuteri,* and *Bifidobacterium* attenuated the production of IL-6, IFN-γ, and nitric oxide by peritoneal cells. TNF-α was not detectable in peritoneal cultures. None of the bacteria altered ex vivo production of cytokines or nitric oxide by Peyer's patch or spleen cell cultures. Taken together, the results suggest that prior oral exposure to lactic acid bacteria could differentially potentiate or attenuate subsequent cytokine and nitric oxide production by peritoneal cells.

Almost a century ago, Metchnikoff *(21)* suggested in his book ''The Prolongation of Life'' that consumption of fermented dairy products resulted in improved health and a longer life. Specifically, lactic acid bacteria associated with fermented dairy products such as cheese, buttermilk, and yogurt are thought to impart these effects. Today, an increasing number of health foods and so-called functional foods as well as pharmaceutical preparations are promoted with health claims based on the probiotic characteristics of some of these bacteria. The most evident effects of probiotics involve changes in viable counts of microorganisms in the intestinal flora after ingestion that can be caused by competition for adhesion sites and nutrients between the ingested microorganisms and potential pathogens *(32).* Another putative mode of action for probiotics is production of antibacterial substances. Lactic cultures may also improve absorbability of certain nutrients, alleviate lactose intolerance symptoms, improve metabolism of some drugs, reduce serum cholesterol, improve intestinal motility, anticancer effects, inactivate bacterial enterotoxins, and stimulate the immune system *(9, 10).*

Understanding the interaction of lactic cultures with the immune system is a formidable task. Humoral and cell-mediated immunity are tightly regulated in the mucosal and systemic immune compartment by several cell types and the cytokines they produce *(7, 31).* The gastrointestinal mucosal immune system is formed by Peyer's patches, lamina propria, and the intestinal wall, whereas the spleen and lymph nodes dispersed throughout the body represent the systemic immune compartment. The intestinal wall contains macrophages and over half of the lymphocytes (T and B cells) present in the body. Even though the peritoneal cavity surrounds the gastrointestinal system, the exact relationship between these two components of the immune system remains unclear.

Cytokines are a diverse group of protein mediators that are produced during the effector phase of an immune response *(1).* Their synthesis is initiated by transcriptional activation of DNA, mRNA production, and translation of this mRNA to proteins (i.e., cytokines). Once synthesized, cytokines are usually secreted as needed, playing a very important function in host immunity. A number of investigations suggest that lactic acid bacteria immunopotentiate the gut mucosal immune system *(23–25, 27–30, 34)* via activation of macrophages and production of cytokines. Increased secretion of interleukin (IL)-1 and tumor necrosis factor (TNF)-α by peritoneal macrophages occurs after intraperitoneal exposure to *Bifidobacterium longum,* or *B. animalis (35)* and *Lactobacillus bulgaricus,* or *Streptococcus thermophilus (36).* In addition to cytokines, leukocytes can produce reactive oxygen intermediates involved in immune response to invading microorganisms. One of the most important of the mediators is nitric oxide.

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TABLE 1. *Cultures used in this study and their sources*

Bacteria	Strain	Source
Bifidobacterium	Bf-1	Sanofi Bio-Industries
L. acidophilus	La-2	Sanofi Bio-Industries
L. bulgaricus 1489	NCK 231	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

The effect of oral administration of lactic acid bacteria on cytokine expression and production needs further clarification. In this study, we hypothesized that cytokine and nitric oxide production in mice are affected by orally administered lactobacilli, streptococci, or bifidobacteria. The objectives of this ex vivo study were to evaluate the effects of oral exposure of mice to viable lactic acid bacteria (*L. acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri, S. thermophilus,* and *Bifidobacterium*) on cytokine and nitric oxide production by mitogen-stimulated Peyer's patch, spleen, and peritoneal cell cultures. These latter effects were further related to nitric oxide production. The results suggested that cytokine and nitric oxide production were differentially affected by the lactic cultures.

MATERIALS AND METHODS

Chemicals and reagents. All inorganic chemicals and organic solvents were reagent grade or better. Neomycin sulfate, paromomycin sulfate, nalidixic acid, lithium chloride, penicillin/streptomycin, RPMI-1640, lipopolysacharide (LPS), bovine serum albumin (BSA), *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED), and sulfanilamide were obtained from Sigma Chemical Co. (St. Louis, Mo.); 2-mercaptoethanol was from Aldrich Chemical Co. (Milwaukee, Wis.); MRS broth, agar, and media were from Difco Laboratories (Detroit, Mich.).

Microorganisms. Cultures of *L. acidophilus, L. bulgaricus, L. casei, L. gasseri, L. helveticus, L. reuteri, S. thermophilus,* and *Bifidobacterium* were obtained from three different sources: American Type Culture Collection (Raleigh, N.C.), Dr. T. R. Klaenhammer (North Carolina State University), and Sanofi Bio-Industries (Waukesha, Wis.) (Table 1). Initially, lactobacilli and streptococci were grown in MRS broth *(5)* while bifidobacteria were grown in MRS + 5% (wt/wt) lactose (MRSL) broth. All organisms were incubated at 37°C for a period of approximately 15 h. A 1% (vol/vol) inoculum was transferred to fresh broth, and this was incubated until entry into log phase (8 to 20 h, depending on the species) at 37°C. Cultures were plated in MRS agar for lactobacilli, modified *Streptococcus thermophilus* (ST) agar (Lee's agar) *(16)* for streptococci, and neomycin sulfate, paromomycin sulfate, nalidixic acid, lithium chloride agar *(37)* for bifidobacteria to determine bacterial counts. Agar plates were incubated at 37°C for 48 h aerobically for lactobacilli and streptococci and anaerobically with Gas Pak (Becton Dickinson Co., Cockesysville, Md.) for bifidobacteria. Colonies were counted using a Quebec counter (Fisher Scientific, Pittsburgh, Pa.). Bacteria were recovered by centrifugation at 1,100 × *g* for 15 min, washed once with a physiological saline solution, and resuspended in 10% (wt/vol) nonfat dry milk (NDM). Doses were aliquoted in single vials containing 0.3 ml 10% NDM with 1.0×10^9 cells and frozen at −80°C for no longer than 3 weeks.

Animal model. Eight-week-old female $B6C3F_1$ mice (six to eight mice per experimental group) were used for ex vivo experiments. All animal handling was conducted in strict accordance with guidelines established by the National Institutes for Health. Experiments were designed to minimize numbers of animals required to test adequately the proposed hypothesis and approved by Michigan State University Laboratory Animal Research committee. Mice were housed three to four per cage in a 24-h light/dark cycle. Water was provided ad libitum. The basic feed was nutritionally complete (AIN-93G) *(33).* Bacteria were orally administered to mice by gavaging a single dose of 1×10^9 cells per mouse in 10% (wt/vol) NDM. NDM was used as the vehicle for bacterial suspensions to simulate dairy food composition as has been done previously by other investigators *(27–29).* Eight hours after administration of the bacteria, blood was collected from anesthetized mice from the retroorbital plexus, peritoneal cells were isolated, and mice were then sacrificed by cervical dislocation. Peyer's patches and spleens were removed and dissociated into single cell suspensions for culturing.

Serum preparation. Serum was obtained from blood after overnight incubation at 4°C and centrifugation at 1,000 × *g* for 15 min. Serum samples were aliquoted and stored at −80°C prior to monitor for immunoglobulins.

Leukocyte cultures. Peritoneal cells were prepared by injecting 2 to 3 ml RPMI-1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) supplemented with 100 U/ml penicillin, 100 μg/ml 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 euthanized mice with a 10-ml syringe fitted with a 20-gauge needle. After gentle massage of the abdomen and without withdrawing the needle, the remaining 7 to 8 ml of medium was injected. Abdomen was massaged again and the liquid was slowly drawn back inside the syringe. After all the fluid was evacuated with the syringe, a small hole was cut in the peritoneum, and the remaining fluid was collected with a sterile Pasteur pipette. The lavage fluid was centrifuged 800 × *g* for 5 to 10 min and the supernatant discarded. Ten milliliters of the same medium was added to resuspend the cells. Cells were counted with a Neubauer hemacytometer.

Peyer's patches were 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 × *g* for 10 min, and resuspended in 2 ml of the same medium and counted. The spleen was removed aseptically, teased apart with tissue forceps in 10 ml 10% FBS–RPMI-1640, and

centrifuged at 450 × *g* for 10 min. Erythrocytes were lysed for 5 min at room temperature in 5 ml of a buffer containing nine parts of 0.16 M ammonium chloride plus one part 0.17 M Tris buffer (pH 7.2). Ten milliliters of fresh medium were added and cells were centrifuged at 450 × *g* for 10 min and counted.

Spleen, Peyer's patch lymphocytes (5×10^5 cells/ml), and peritoneal cells (1.5×10^5 cells/ml) were cultured in 24-well tissue culture plates with 10% FBS–RPMI-1640 medium in the absence (unstimulated) and in the presence of inducing agents such as LPS (1 μg/ml) from *Salmonella* Typhimurium, phorbol 12-myristate-13 acetate (PMA; 10 ng/ml), and ionomycin (I; 0.5 μg/ml). The tissue culture supernatants were collected after 2 and 5 days and monitored for cytokines (IL-12, IL-6, IFN-γ, TNF-α), by enzyme-linked immunosorbent assay (ELISA) and nitric oxide.

Cytokine quantitation. Cytokines were quantitated in supernatants by ELISA. Immunolon 4 Removawell microtiter strips (Dynatech Laboratories Inc., Chantilly, Va.) were coated overnight at 4°C with 50 μl/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 [IL-6, TNF-α], 2 μg/ml [IFN-γ], or 0.5 mg/ml [IL-12]). Plates were washed three times with 0.01 M phosphate-buffered (pH 7.2) saline (PBS) containing 0.2% Tween 20 (PBS-T). Plates were blocked with 300 μl of 3% (wt/vol) BSA in PBS (BSA–PBS) at 37°C for 30 min, and washed three times with the 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 four times with PBS-T, and 50 μl of biotinylated rat anti-mouse cytokine detection monoclonal antibody (1.5 μg/well; PharMingen) diluted in BSA–PBS was added to each well. Plates were incubated at room temperature for 60 min and washed six times with PBS-T and one additional time with distilled water. Fifty microliters of streptavidin–horseradish peroxidase conjugate (1.5 μg/well; Sigma) diluted in BSA–PBS was 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., Menlo Park, Calif.) and cytokine concentrations were quantitated by using Vmax Software.

Nitric oxide production. Cells were cultured as described above for 2 and 5 days, and nitric oxide production was assessed by measuring nitrite accumulation, a stable metabolic product of nitric oxide, in the culture supernatants. Nitrite concentrations were determined by the Griess reaction *(8).* Briefly, equal amounts of NED (100 mg dissolved in 100 ml of distilled water) and sufanilamide (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 using the Vmax kinetic microplate reader (Molecular Devices Co.), and nitrite concentrations were quantitated by using Vmax Software.

Statistical methods. Data were summarized with descriptive statistics such as the mean and standard error of the mean. Statistical comparisons were analyzed using a Student's *t* test for comparison between two groups or by Dunnett's test following one-way analysis of variance (ANOVA) for several groups using the SigmaStat System (Jandel Scientific, San Rafael, Calif.). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

The effects of exposure to isolates of *L. acidophilus, L. bulgaricus, L. casei, L. helveticus, L. gasseri, L. reuteri,* and *S. thermophilus* in mice on cytokine production were assessed in mitogen-stimulated and unstimulated leukocyte cultures. Mice were fed a single dose of a microbe suspension and sacrificed after 8 h. Peyer's patch and spleen cells were isolated and peritoneal cells were isolated and cultured with or without mitogens (LPS or PMA + I) for 2 and 5 days. LPS induces activation of macrophages and antigen-presenting cells *(3).* PMA is an activator of protein kinase C while I is a calcium ionophore that increases the levels of intracellular calcium *(38)*; together they strongly enhance T-cell activation. TNF-α production was not detectable in peritoneal cultures. Lactic acid bacteria did not affect cytokine (TNF-α, IL-6, IL-12, and IFN-γ) or nitric oxide production in Peyer's or spleen patch cultures.

IL-6 was produced in considerable amounts by peritoneal cells from mice fed NDM or cultures (Fig. 1). Effects on cytokine production by peritoneal cells were strain dependent. Prior *L. casei* ATCC 39539 and *L. acidophilus* La-2 exposure stimulated IL-6 production in peritoneal cells cultured without mitogen. LPS-treated cells produced the highest IL-6 levels, with *L. casei* ATCC 39539 having a potentiative effect. IL-6 was also produced by peritoneal cells from mice fed *L. helveticus* Lr-92, *L. gasseri* ADH (NCK 101), *L. reuteri* ATCC 23272, and *Bifidobacterium* Bf-1 when cells were stimulated with LPS, but synthesis of this cytokine was attenuated when these same cells were stimulated with PMA + I.

Peritoneal cells stimulated with PMA + I but not LPS showed marked IFN-γ production (Fig. 2). Those cells coming from mice fed *L. bulgaricus* 1489 (NCK 231), *S. thermophilus* St-133, *L. casei* ATCC 39539, and *L. acidophilus* La-2 produced equivalent or greater IFN-γ when compared to the control group fed NDM. In contrast, those cells coming from mice treated with *L. helveticus* Lr-92, *L. gasseri* ADH (NCK 101), *L. reuteri* ATCC 23272, and *Bifidobacterium* Bf-1 exhibited markedly depressed IFN-γ production as was observed for IL-6.

Unstimulated peritoneal cells from mice exposed to *L. casei* and *L. acidophilus* produced significantly more IL-12p40 than the NDM control (Fig. 3). Peritoneal cells produced more IL-12p40 when stimulated with LPS than when stimulated with PMA + I, and this was attenuated by *L. casei.* LPS-induced IL-12p40 was also increased in peritoneal cells isolated from mice exposed to *L. acidophilus.* However, effects on IL-12p40 by other lactic cultures were not consistent. IL-12p35 was undetectable in all cases.

Nitric oxide production was relatively low after incubation of peritoneal cells for 2 days (Fig. 4). After 5 days,

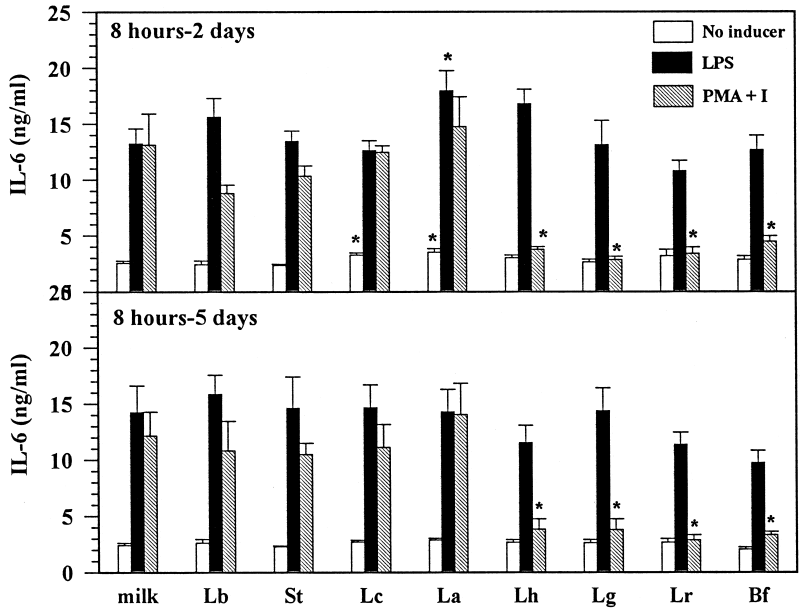

FIGURE 1. *IL-6 levels in peritoneal cell (1.5 × 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Peritoneal cells were obtained 8 h after administration of lactic acid bacteria as described in the Materials and Methods and cultured in the absence and in the presence of inducing agents (LPS, 1 μg/ml; PMA, 10 ng/ml + I, 0.5 μg/ml). Culture supernatants were collected after 2 and 5 days and assayed for IL-6 by ELISA. Lb,* L. bulgaricus *1489 NCK 231; St,* S. thermophilus *St 133; Lc,* L. casei *ATCC 39539; La,* L. acidophilus *La 2; Lh,* L. helveticus *Lr 92; Lr,* L. reuteri *ATCC 23272; Bf,* Bifidobacterium *Bf 1. Data are mean ± SEM of duplicate cultures (*n = 6*) and are representative of two separate experiments. * Indicates significant differences with respect to the control group fed NDM (*P ≤ 0.05*).*

levels of nitric oxide increased for those cells stimulated with PMA + I, following a pattern very similar to the one presented for IFN-γ production. Here, *L. acidophilus* potentiated the response, whereas *L. helveticus* Lr-92, *L. gasseri* ADH (NCK 101), *L. reuteri* ATCC 23272, and *Bifidobacterium* Bf-1 inhibited production of this mediator. *S. thermophilus, L. casei, L. acidophilus, L. reuteri, and Bifidobacterium* exposure impaired nitric oxide production in LPS-stimulated cultures.

DISCUSSION

Although several studies have reported the in vitro effects of lactic acid bacteria on cytokine production *(6, 18, 19, 35),* very little information is available about cytokine production in vivo. In this study, we used an ex vivo strategy to determine effects of oral administration of lactic acid bacteria on cytokine production by cultured leukocytes. The main finding of our study is that prior exposure to certain

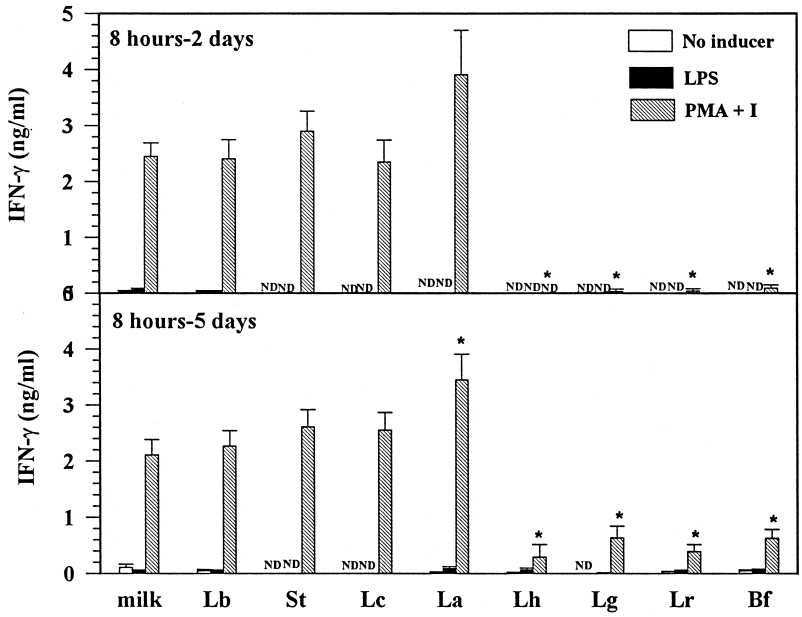

FIGURE 2. *INF-γ levels in peritoneal cell (1.5 × 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Conditions and analysis were as described in Figure 1.*

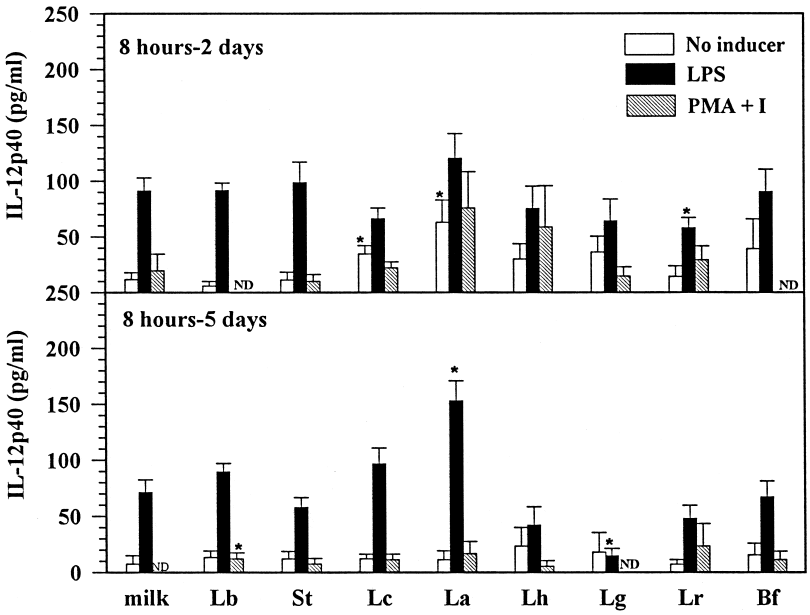

FIGURE 3. *IL-12p40 levels in peritoneal cell (1.5 × 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Conditions and analysis were as described in Figure 1.*

lactic acid bacteria altered subsequent mitogen-induced cytokine and nitric oxide production by peritoneal cells, but production of these mediators was not affected in mucosal or systemic leukocytes derived from Peyer's patches and spleen, respectively.

This study examined the effect on peritoneal cells using two different mitogenic signals. One signal was provided by LPS from *Salmonella* Typhimurium, which is a macrophage activator *(3)* at the concentration (1 μg/ml) employed here. Also, a combination of PMA, which activates protein kinase C, and I, which increases intracellular calcium [Ca^{2+}] *(38)*, was used. These two compounds can affect both macrophages and T cells. The peritoneal cavity is frequently used as a concentrated source of mature murine macrophages. Peritoneal lavage yields 2 to 8 × 10^6 cells of which 20 to 40% are macrophages, but other cell types are also present *(14)*. B cells represent 10 to 40% *(14, 15)* of the population, while T cells and other type of cells such as natural killer (NK cells) may represent at least another 20%. Thus, other cells besides macrophages may contribute to the findings observed in peritoneal cell cultures.

The spectrum of products observed in our ex vivo study with peritoneal cells suggests that macrophages are a key target for lactic acid bacteria immunomodulation activity. The effect of various strains relative to their capacity to secrete IL-6, IL-12, and nitric oxide ranged from stim-

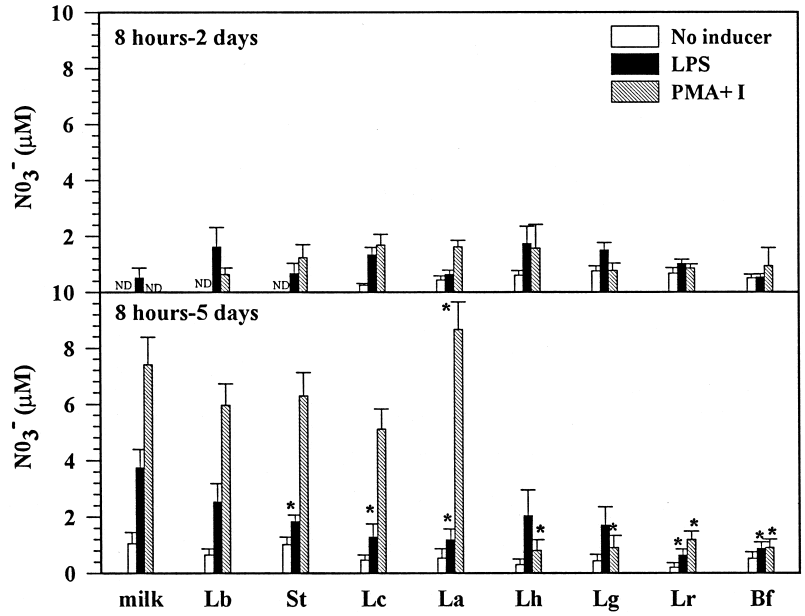

FIGURE 4. *Nitrate levels in peritoneal cell (1.5 × 10^5 cells/ml) cultures from mice fed a single dose of lactic acid bacteria. Conditions and analysis were as described in Figure 1.*

ulation to inhibition. The potential to alter macrophage function is very important for several reasons. Macrophages constitute the second major cell population of the immune system with phagocytosis being a primary function. They originate in bone marrow, and, after migration, and maturation, they settle in tissues as mature macrophages *(13).* These can be activated by a variety of stimuli, and their principal functions include phagocytosis of foreign particles, antigen presentation, and production of cytokines (IL-6, TNF-α, IL-1, IL-12) or reactive oxygen mediators, such as nitric oxide, that recruit other inflammatory cells. Thus, macrophages can participate both in humoral and cell-mediated immune responses.

IL-6 is a pleiotropic cytokine that can affect T cells, B cells and macrophages. IL-12 is a heterodimer and its principal biologic effect is upregulation of the Th1 response via induction of IFN-γ production, T-cell proliferation, and NK cell-mediated cytotoxicity *(12).* IFN-γ activates macrophages, and these activated macrophages are able to produce TNF-α, IL-6, IL-12, oxygen radicals, and nitric oxide *(13).* Nitric oxide is produced by immune cells in response to cytokines such as TNF-α, IFN-γ, and IL-2 *(17)* by stimulating the expression of inducible nitric oxide synthase and acting in combination with LPS in murine macrophages. Nitric oxide also may participate in inflammatory and autoimmune-mediated tissue destruction *(41).*

Several authors have similarly reported that macrophage activation occurs after lactic acid bacteria administration. Saito et al. *(34)* found that intramuscular injection to mice with heat-killed *L. casei* LC 99018 increased macrophage function. Perdigon et al. *(27)* observed an enhanced macrophage and lymphocytic activity by administering a mixed culture of *L. acidophilus* ATCC 4356 and *L. casei* CRL 431 to mice. This same group later reported a similar conclusion in a feeding study with *L. casei* and *L. acidophilus* using mice infected with a lethal dose of *Salmonella* Typhimurium *(25). L. casei* and *L. bulgaricus* were also studied and found to activate peritoneal macrophages in mice when given either orally or intraperitoneally *(28).* These authors suggested that the bacteria, when passing through the intestinal tract, may be responsible for enhancing host immune response. *L. bulgaricus* was generally less effective than *L. acidophilus* in increasing the activity of peritoneal macrophages as measured by phagocytic assay. Similar results were obtained when using *S. thermophilus* and *L. acidophilus (29)* or fermented milks with *L. casei, L. acidophilus,* and/or a mixture of both *(24, 26, 30).*

Surprisingly, neither peritoneal cells nor spleen or Peyer's patch lymphocytes appeared to respond to LPS or PMA + I by producing TNF-α, a typical cytokine produced by activated macrophages. Studies of the kinetic properties on the transcription of mRNA and secretion of IL-6, IL-1, and TNF-α showed that TNF-α mRNA transcription and protein production occur very rapidly *(20).* TNF-α mRNA peaks 1 to 2 h after the stimulus was produced, and the cytokine can be collected in supernatants after 2 to 4 h culture. IL-6 and IL-1 mRNA peak after 4 to 8 h and simultaneously, but the protein is present at the highest concentration between 8 and 12 h for IL-6 and 12 h for IL-1. Thus, it is possible that we missed detecting TNF-α after 2 or 5 days.

Our study suggests that besides macrophages, other types of cells were affected by lactic acid bacteria as indicated by the production of IFN-γ. IFNs are proteins with a variety of physiological effects. They are produced mainly by T lymphocytes, macrophages, and NK cells. IFN-α and -β are closely related and produced by T cells and macrophages. NK cells are large lymphoid cells with many intracellular granules that are able to recognize and kill abnormal cells even though they do not bear antigen-specific receptors. IFN-γ is produced exclusively by T lymphocytes and NK cells, whereas macrophages do not express the IFN-γ gene *(13).* Lactic cultures have also been previously shown to stimulate IFN-γ production. Halpern et al. *(11)* reported a rise in serum IFN-γ production in young human adults when they consumed two cups of yogurt daily. *L. bulgaricus* and *S. thermophilus* added to human peripheral blood lymphocytes have an adjuvant action, potentiating IFN-γ production *(6).*

Peritoneal cells were used in the study because they contain large numbers of macrophages that are easily amenable to isolation. Because they are loosely adherent, collection of peritoneal macrophages do not require mechanical disaggregation or enzymatic digestion as would be necessary for other tissues. Although their exact role within the immune system is unclear, leukocytes in the peritoneal cavity provide immediate immune protection against invading microbes that may penetrate the abdominal area due to intestinal injury or damage. Bellingan et al. *(2)* recently showed that macrophages migrate from the peritoneum to the draining lymph nodes. The migration process takes more than 4 h, but once started, these macrophages are cleared within 96 h from the peritoneum. Thus, macrophages coming from the peritoneal cavity may have enhanced capability to respond to invading microbes as a consequence of lactic acid bacteria and NDM administration. This may be apparent both within the peritoneum and perhaps in other lymphoid tissues after migration.

It should be noted that vehicle plus dietary factors may influence the basal capacity to produce cytokine in control groups in this study. Casein is a dietary component present in NDM and in murine diet AIN-93G, which could be capable of producing immune responses in fed-NDM groups. Peptides derived from casein were shown to contain an immune-activating factor *(42).* Diets containing between 20 and 40% of casein increase cellular immunity in spleen cells and macrophages *(39).*The casein fraction is composed of different proteins (α, β, and κ). Notably, κ casein triggers lymphocyte proliferation *(40).* Also, β-casein promotes cellular and humoral responses *(4).* During enzymatic digestion of human and bovine caseins, peptides are formed and released that are able to stimulate phagocytic activity of murine and human macrophages, and these apparently exert in vivo a protective effect against infections *(22).*

The research presented herein suggests that lactic acid bacteria can alter peritoneal cell function, and this might subtly impact immunity. On a speculative level, these effects could be mediated by the bacterial cell wall or cyto-

plasmic components that affect immune cells via receptors present on the macrophage cell surface and perhaps other cells such as NK cells. However, it must be recognized that the ex vivo model described here has limitations with respect to duplicating in vivo effects. Furthermore, it should be recognized that because the mouse has different intestinal flora and diet than humans, extension of the results to the human immune system must be done with caution. Thus, further mechanistic understanding is warranted on how the other specific immunologic effects described in this and other studies are mediated by lactic acid bacteria.

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REFERENCES

1. Abbas, A. K., A. H. Lichtman, and J. S. Pober. 1994. Cellular and molecular immunology, 2nd ed. Saunders Co., Philadelphia, Pa.
2. Bellingan, G. J., H. Caldwell, S. E. M. Howie, I. Dransfield, and C. Haslett. 1996. In vivo fate of the inflammatory macrophage during the resolution of inflammation. J. Immunol. 157:2577–2585.
3. Beutler, B., and V. Krays. 1995. Lipopolysaccharide signal transduction, regulation of tumor necrosis factor biosynthesis and signaling by tumor necrosis factor itself. J. Cardiol. Pharmacol. 25(suppl. 2): S1–S8.
4. Cavallo, M. G., D. Fava, L. Monetini, F. Barone, and P. Pozzilli. 1996. Cell-mediated immune response to beta-casein in recent onset insulin-dependent diabetes: implications for disease pathogenesis. Lancet 348(9032):926–928.
5. 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.
6. De Simone, C., B. B. Salvadori, R. Negri, M. Ferrazzi, L. Baldinelli, and R. Vesely. 1986. The adjuvant effect of yogurt on production of gamma-interferon by con-A stimulated human peripheral blood lymphocytes. Nutr. Rep. Int. 33:419–433.
7. de Vos, T., and T. A. Dick. 1991. A rapid method to determine the isotype and specificity of coproantibodies in mice infected with *Trichinella* or fed cholera toxin. J. Immunol. Methods 141:285–288.
8. 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.
9. Fuller, R. 1991. Probiotics in human medicine. Gut 32:439–442.
10. Gilliland, S. E. 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. 87:175–188.
11. 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.
12. Hendrzak, J. A., and M. J. Brunda. 1995. Interleukin 12 biologic activity, therapeutic utility and role in disease. Lab. Invest. 72:619–637.
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, S. H., T. H. Kim, and D. V. Han. 1996. The main peritoneal source of precursors of the murine intestinal IgA plasma cell. Anticancer Res. 16:2821–2824.
15. Kroese, F. G. M., E. C. Butcher, A. M. Stall, P. A. Lalor, S. Adams, and L. A. Herzenberg. 1989. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the perioneal cavity. Int. Immunol. 1:75–84.
16. Lee, S. Y., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1974. An agar medium for the differential enumeration of yogurt starter bacteria. J. Milk Food Technol. 37:272–278.
17. Lin, H., R. Q. Wei, and S. F. Bolling. 1995. Tumor necrosis factor-alpha and interferon gamma modulation of nitric oxide and allograft survival. J. Surg. Res. 59:103–110.
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. Murtha, Z. Ustunol, and J. Pestka. 1997. Effects of *Lactobacillus* spp. on cytokine production by RAW 2;64.7 macrophage and EL-4 thymoma cell lines. J. Food Prot. 60:1364–1370.
20. Martin, C. A., and M. E. Dorf. 1990. Interleukin-6 production by murine macrophage cell lines P388D1 and J774A.1: stimulation requirements and kinetics. Cell. Immunol. 128:555–568.
21. Metchnikoff, E. 1907. The prolongation of life. G. P. Putnam and Sons, The Knickerbocker Press, New York.
22. Migliore-Samour, D., F. Floc'h, and P. Jolles. 1989. Biologically active casein peptides implicated in immunomodulation. J. Dairy Res. 56:357–362.
23. Perdigon, G., S. Alvarez, N. Gobbato, M. V. Budeguer, and A. Pesce de Ruiz Holgado. 1995. Comparative effect of the adjuvant capacity of *Lactobacillus casei* and lipopolysaccharide on the intestinal secretory antibody response and resistance to *Salmonella* infection in mice. Food Agric. Immunol. 7:283–294.
24. Perdigon, G., S. Alvarez, and M. Medici. 1992. Systemic and local augmentation of the immune response in mice by feeding with milk fermented with *Lactobacillus acidophilus* and/or *Lactobacillus casei.* Dyn. Nutr. Res. 1:66–76.
25. Perdigon, G., S. Alvarez, M. E. Nader de Macias, M. E. Roux, and A. Pesce de Ruiz Holgado. 1990. The oral administration of lactic acid bacteria increase the mucosal intestinal immunity in response to enteropathogens. J. Food Prot. 53:404–410.
26. Perdigon, G., M. E. de Jorrat, F. de Petrino, and M. Valverde de Budeguer. 1991. Effect of oral administration of *Lactobacillus casei* on various biological functions of the host. Food Agric. Immunol. 3:93–102.
27. 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. 49:986–989.
28. Perdigon, G., M. E. Nader de Macias, S Alvarez, G. Oliver, and A. Pesce de Ruiz Holgado. 1986. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect. Immu. 53:404–410.
29. Perdigon, G., M. E. Nader de Macias, S Alvarez, G. Oliver, and 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.
30. Perdigon, G., M. E. Nader de Macias, S Alvarez, G. Oliver, and A. Pesce de Ruiz Holgado. 1988. Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophilus.* Immunology 63:17–23.
31. Pestka, J. J. 1993. Food, diet and gastrointestinal immune function, p. 1–66. *In* J. E. Kinsella (ed.), Advances in food and nutrition research, vol. 37. Academic Press, San Diego, Calif.
32. Rafter, J. J. (1995). Review: the role of lactic acid bacteria in colon cancer prevention. Scand. J. Gastroenterol. 30:497–502.
33. Reeves, P. G., F. H. Nielsen, and G. C. Fahey. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123:1939–1951.
34. 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.
35. 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 13:79–89.

36. Solis Pereyra, B., R. Falcoff, E. Falcoff, and D. Lemonnier. 1991. Interferon induction by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in mice. Eur. Cytokine Network 2:299–303.
37. Teraguchi, S., M. Uehara, K. Ogasa, and T. Mitsuoka. 1978. Enumeration of bifidobacteria in dairy products. Jpn. J. Bacteriol. 33: 753–756.
38. Truneh, A., F. Albert, P. Golstein, and A. M. Schmitt-Verhulst. 1985. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 313:318–320.
39. Ueda, N., J. Kayashita, S. Moriguchi, and Y. Kishino. 1990. Effect of dietary protein levels on immune function of exercised rats. Nutr. Res. 10:429–437.
40. Werfel, T., G. Ahlers, P. Schmidt, M. Boeker, and A. Kapp. 1996. Detection of a kappa-casein-specific lymphocyte response in milk-responsive atopic dermatitis. Clin. Exp. Allergy 26:1380–1386.
41. Yamashita, M., H. Niki, M. Yamada, S. Mue, and K. Ohuchi. 1997. Induction of nitric oxide synthase by lipopolysaccharide and its inhibition by auranofin in RAW 264.7 cells. Eur. J. Pharmacol. 338: 151–158.
42. Yamauchi, F., and K. Suetsuna. 1997. Immunological effects of dietary peptide derived from soybean protein. J. Nutr. Biochem. 4: 450–457.