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Research Note

Effects of Lactic Acid Bacteria Ingestion on Basal Cytokine mRNA and Immunoglobulin Levels in the Mouse

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ABSTRACT

An increasing number of functional foods and pharmaceutical preparations containing lactic acid bacteria are being promoted with health claims based on the potential probiotic characteristics and on their capacity for stimulating the host immune system. However, the specific immune effects of oral administration of these microbes remain undefined. In this study, we tested the hypothesis that basal gastrointestinal immune status in mice is affected by orally administered lactic acid bacteria. The specific objective of this research was to evaluate the effects of repeated oral exposure to viable and nonviable lactic acid bacteria (*Lactobacillus acidophilus, L. bulgaricus, L. casei,* and *Streptococcus thermophilus*) in mice on basal cytokine mRNA expression in mucosal (Peyer's patches), systemic (spleen), and lymphoid tissue and on immunoglobulin levels. The results indicated that oral exposure to 10^9 CFU/day for up to 14 days did not significantly affect basal interferon-γ, tumor necrosis factor-α, or interleukin-6 mRNA expression or total serum and intestinal immunoglobulins.

Almost a century ago, Metchnikoff *(16)* suggested in his book ''The Prolongation of Life'' that consumption of dairy products fermented by lactic acid bacteria resulted in improved health and a longer life. 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 competition for adhesion sites and nutrients between the ingested microorganisms and potential pathogens *(25).* Other health effects attributed to lactic cultures are improved absorbability of certain nutrients, alleviation of lactose intolerance symptoms, serum cholesterol reduction, improvement of intestinal motility, anticancer effects, inactivation of enterotoxins from pathogens, and stimulation of the immune system *(6, 7).*

The immune system is tightly regulated by several leukocyte types and the proteins they produce *(15).* In general, cytokines are considered a diverse group of protein hormones produced through the effector phase of an immune response, whereas another group of proteins, the immunoglobulins, are involved in humoral immunity *(1).* The effect of oral administration of lactic acid bacteria on expression of these mediators is not yet well defined.

A number of investigations suggest that lactic acid bacteria potentiate the gastrointestinal immune function system *(18–23, 27).* The gut mucosal immune system is a critical site of host defense *(9)* that includes Peyer's patches and lamina propria, while spleen and lymph nodes dispersed along the body represent the systemic immune compartment. The intestinal wall contains macrophages and over half of the lymphocytes (T and B cells) present on the body, many of which produce cytokines (interferon [IFN]-γ) *(24).* Differentiated B cells that produce mainly IgA are located in the Peyer's patches and lamina propria in the small intestine or travel to spleen using the systemic circulation. Secretory IgA acts by binding microbes and toxins and is a critical component of the gut immune defense system.

In this study, we hypothesized that gastrointestinal cytokine gene expression and immunoglobulin production in mice are affected by orally administered lactic acid bacteria. The objectives of this study were to evaluate the effects of in vivo exposure to viable and nonviable strains of lactic acid bacteria (*Lactobacillus acidophilus, L. bulgaricus, L. casei,* and *Streptococcus thermophilus*) on cytokine mRNA gene expression in mucosal and systemic lymphoid tissue as well as immunoglobulin production in the mouse.

MATERIALS AND METHODS

Microorganisms. The microorganisms used in this study were kindly supplied by Dr. T. R. Klaenhammer (North Carolina State University) (*L. bulgaricus* 1489 NCK 231) and Michelle Malone (Sanofi Bio-Industries, Waukesha, Wis.) (*L. bulgaricus* Lr 78, *L. bulgaricus* Lr 79, *L. bulgaricus* Lr 28; *L. acidophilus* La 1, *L. acidophilus* La 7, *L. acidophilus* La 2; *S. thermophilus* St 133), or purchased from the American Type Culture Collection (Raleigh, N.C.) (*L. acidophilus* 53545, *L. acidophilus* 521, *L. casei* 39539, *L. casei* 334). Initially, lactobacilli and streptococci were grown in MRS broth (Difco Laboratories, Detroit, Mich.) and incubated at 37°C for a period of approximately 15 h. A 1% (vol/vol) inoculum was transferred to fresh broth and incubated at 37°C

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until entry into log phase (8 to 20 h, depending on the species). Total cell numbers were estimated by plating cultures in MRS agar for lactobacilli and modified ST agar (Lee's agar) *(11)* for streptococci at 37°C for 48 h aerobically. Some bacteria cultures were heat killed at 100°C for 50 min. Viable and nonviable (i.e., heated) cultures were centrifuged at 1,500 × *g* for 15 min to recover bacteria, washed once with a physiological saline solution, and resuspended in 10% (wt/vol) nonfat dry milk (NFDM). Doses were aliquoted in single vials containing 0.3 ml 10% NFDM with 1.0×10^9 cells and frozen at −80°C for no longer than 3 weeks. Viability during the course of one study was verified by plating a single dose every 5 days during 15 consecutive days, using agar media and conditions described above.

Animal model. Eight-week-old female $B6C3F_1$ mice (six to eight mice per experimental group) were used for in vivo experiments. Mice were housed three to four per cage in a 24-h light/dark cycle. Water was provided ad libitum. The basic feed was a nutritionally complete semipurified diet (AIN-93G) *(26).*

Lactic acid bacteria administration. Bacteria were orally administered as suspensions in 10% (wt/vol) NFDM by gavaging doses of 1.0×10^9 cells per mouse per day. Matching controls were fed 10% (wt/vol) NFDM without organisms. At 1, 7, 14 days, and 2 h after the last dose was administered, blood was collected, and mice were sacrificed by cervical dislocation after gentle anesthesia for extraction of Peyer's patches, mesenteric lymph node, and spleen.

Serum preparation. Blood was collected from anesthetized mice from the retroorbital plexus. Serum was obtained 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 assay for immunoglobulins.

Fecal sample preparation. Fecal samples were prepared as described by de Vos and Dick *(4).* Briefly, feces were collected, aseptically weighed, and placed into centrifuge tubes. Ten milliliters of 0.01 M phosphate-buffered saline (PBS) per gram of feces (vol/wt) were added, and the mixture was incubated for 15 min at room temperature. Samples were mixed by vortexing until suspended, left to settle for 15 min, mixed again, and centrifuged at 22,000 × *g* for 10 min in a Sorval RC 5C centrifuge (Du Pont Co., Wilmington, Del.). The supernatant was removed and stored at −80°C for immunoglobulin measurement.

Immunoglobulin quantitation. IgA and IgG were quantitated in sera and fecal samples by enzyme-linked immunosorbent assay (ELISA) as described by Dong et al. *(5).*

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) for cytokines. Total RNA was extracted from Peyer's patches, spleen, and mesenteric lymph node using the RNA STAT-60 isolation reagent (TEL-TEST "B" Inc., Friendswood, Tex.) according to the manufacturer's instructions. Total RNA content was determined spectrophotometrically at 260 nm *(28).* RT reaction for first-strand cDNA synthesis was carried out by the method of Kawasaki *(10).* Cytokines were detected by the semiquantitative RT-PCR method of Svetic et al. *(31)* using the PCR primers and probes for DNA hybridization as described by Zhou et al. *(32).* PCR was performed in a final volume of 50 μl using a 9600 Perkin-Elmer Cycler (Perkin-Elmer Corporation, Norwalk, CT) with a 5-min incubation step at 95°C for denaturation, followed by a three-step temperature cycling (1 min denaturation at 95°C, 1 min primer annealing at 52°C, and 3 min extension at 72°C) terminated by a 72°C incubation for 10 min then cooled to 4°C. The cycles were repeated for an optimized number for each transcript (15, 20, 24, and 25 cycles for β2-microglobulin [β2-MG], tumor nerosis factor [TNF]-α, IFN-γ, and interleukin [IL]-6, respectively). β2-MG was used as a housekeeping gene to verify initial equal quantities of RNA and the integrity of the RNA preparation.

Hybridization analyses were carried out to determine the relative abundance of PCR-amplified cDNAs using previously described protocols *(2, 32).* Briefly, PCR products (10 μl) were treated with 1 N NaOH and 0.2 M EDTA, boiled for 10 min, and applied to a Nytron membrane (Nytran, Schleicher & Schuell Inc., Keene, N.H.) using a Bio-dot SF microfiltration apparatus (Bio-Rad Laboratories, Hercules, Calif.). Hybridizations employed ^{32}P-labeled probes prepared using a DNA 3′-end labeling system that uses terminal deoxyribonucleotide transferase (TdT) (Promega Co., Madison, Wis.). All oligoprobes had at least a specific activity of 1×10^9 cpm/μg, and all hybridizations were performed with no less than 1×10^7cpm/ml. Blots were exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, N.Y.) with an intensifying screen at −80°C for variable length periods depending upon signal strength. For the purposes of comparison, exposure periods for each cytokine were identical among untreated and treated groups. Autoradiography bands were quantified with an Epson ES-1000C scanner and Sigma-Scan program (Jandel Scientific, San Rafael, Calif.). Relative amounts of cytokine mRNA were estimated by dividing the densitometric area of the cytokine autoradiography band by the densitometric area of the housekeeping gene β2-MG autoradiography band.

Statistical methods. Statistical comparisons of treatment and control groups were analyzed using a Student's *t* test for comparison between two groups using the SigmaStat Statistical Analysis System (Jandel Scientific). A *P* value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

To assess potential immune modulation of bacteria feeding, basal cytokine mRNA expression in spleen, Peyer's patches, and mesenteric lymph node were evaluated after administration of lactic acid bacteria for 1 and 14 days (1×10^9 cells per day). Figure 1 represents a typical result of exposure to viable and heated lactic acid bacteria (*L. bulgaricus* 1489 [NCK 231] in this case) for basal expression of IFN-γ and TNF-α mRNA. In spleen, basal expression of IFN-γ and TNF-α mRNA was higher than in Peyer's patches for all groups. No statistically significant difference was found between treatment and controls of the species tested (*L. casei* ATCC 39539 and *L. bulgaricus* 1489 [NCK 231]) regardless of whether viable or nonviable bacteria were used.

When *L. bulgaricus* 1489 (NCK 231) and *S. thermophilus* St-133 were administered for 14 days (1×10^9 cells/day), significant induction or inhibition was not observed for the basal mRNA for IL-6, IFN-γ, or TNF-α tested in either spleen or Peyer's patches (Fig. 2). These results typify those obtained from three separate experiments not only for the two aforementioned organisms but also for *L. bulgaricus* 1489 (NCK 231), *S. thermophilus* St-133, *L. casei* ATCC 39539, and *L. acidophilus* La-2 (data not shown).

Analysis of sera revealed no statistically significant difference in either total IgA or IgG levels from mice fed *L. acidophilus, L. bulgaricus, L. casei,* and *S. thermophilus*

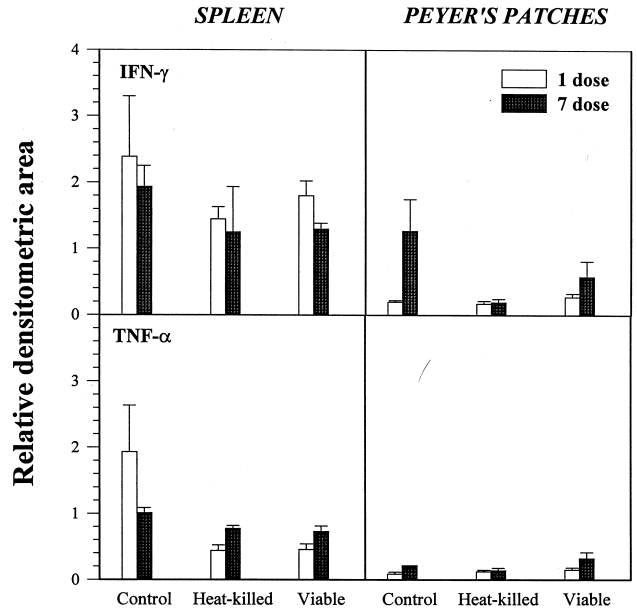

FIGURE 1. *Cytokine mRNA levels in mice after oral exposure to viable and heat-killed* Lactobacillus bulgaricus *1489 (NCK 231) (1.0 × 10^9 cells/mouse/day) for 1 and 7 days. Data are mean ± SEM (*n = 6*) and are representative of two separate experiments.*

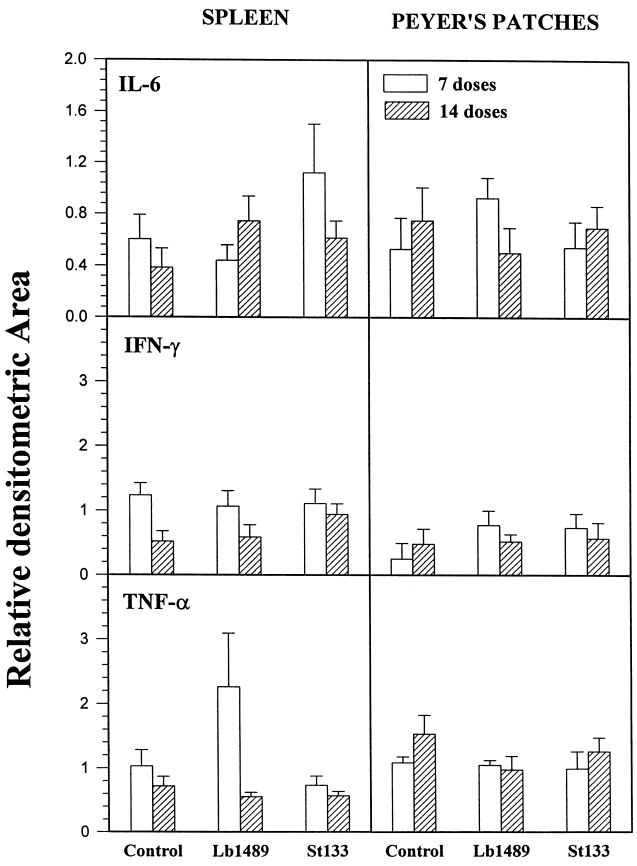

FIGURE 2. *Cytokine mRNA levels in mice after oral exposure to* Lactobacillus bulgaricus *1489 (NCK 231) (Lb1489) and* Streptococcus thermophilus *St 133 (St133) (1.0 × 10^9 cells/mouse/day) for 7 and 14 days. Data are mean ± SEM (*n = 6*) and are representative of three separate experiments.*

(Fig. 3) when compared to control mice fed with NFDM. Similar results were obtained when analyzing for copro-antibodies (Fig. 4).

This study was an attempt to determine potential in vivo effects of oral administration of lactic acid bacteria on basal cytokine gene expression and immunoglobulin levels in mucosal and systemic sites. The main findings were that (i) basal mRNA expression of IL-6, IFN-γ, and TNF-α were not affected by repeated lactic acid bacteria administration, and (ii) immunoglobulin production was similarly unaffected.

We chose to measure basal cytokine mRNA in spleen, Peyer's patches, and mesenteric lymph node because they represent the systemic and mucosal immune compartments. The gut immune response is induced in the Peyer's patches where activation, switching, proliferation, and differentiation of B cells is under the control of T cells, cytokines, and other accessory cells *(17).* As an initial approach we chose to analyze basal cytokine mRNA instead of serum cytokine levels because the short half life of some cytokines makes their quantitation a poor indicator of cytokine production in vivo and because uptake of cytokines by some receptors makes it difficult to measure these proteins accurately. We chose a very sensitive technique, RT-PCR, which includes amplification of mRNA present on a tissue sample for a desired cytokine *(31).* This technique has advantages over Northern analysis because it is much more sensitive and requires very little sample. Limitations of this method are that it requires extensive technical expertise to conduct and also necessitates optimization with regard to number of cycles as well as primer and probe design. Because of the high sensitivity of this method and the inherent variability of general immune status among individuals and populations of mice, basal levels of cytokine mRNAs may vary extensively within a single experiment and among different experiments. Thus, it may be difficult to discriminate very small differences that may occur as a result of exposure to lactic acid bacteria.

It is well established that lactic acid bacteria can stimulate cytokine production in vitro. For example, *L. bulgaricus* and *S. thermophilus* added to human peripheral blood lymphocytes have an adjuvant action, potentiating IFN-γ production *(3).* Increased secretion of IL-1 and TNF-α by peritoneal macrophages occurs after intraperitoneal exposure to *Bifidobacterium longum* or *B. animalis (30).* Other studies conducted by this laboratory showed that whole nonviable lactic acid bacteria cells *(12–14)* stimulate macrophages in vitro to release cytokines. In contrast to in vitro studies, the results presented herein suggest that ingestion of lactic acid bacteria cultures had no effect on basal cytokine gene expression. In an earlier study, we have observed that ingestion of viable and heat-treated yogurt has no effect or actually decreases specific cytokine mRNAs in mucosal and systemic organs *(8).* The in vivo results may differ from previous in vitro studies because the lactic acid bacteria tested here may not survive digestion or have sufficient contact time with the mucosal immune system. Thus it is difficult to make predictions about lactic acid bacteria immunopotentiation based only on in vitro research. Fur-

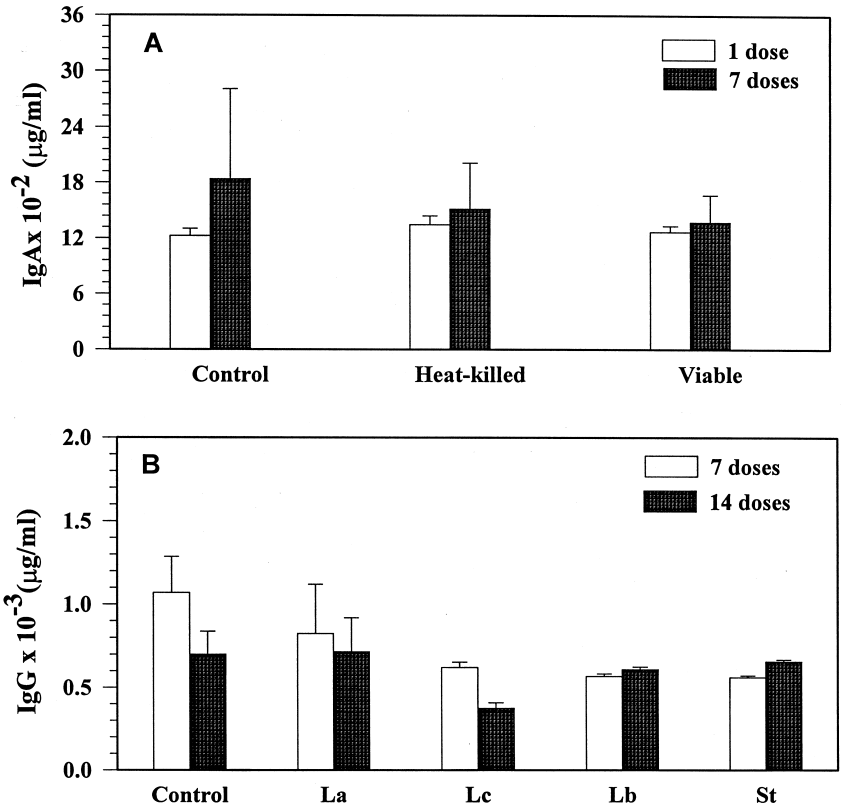

FIGURE 3. *(A) IgA levels in sera collected from mice after oral exposure to viable and heat-killed* Lactobacillus bulgaricus *1489 (NCK 231) (1.0 × 10⁹ cells/mouse/day) for 1 and 7 days. Data are mean ± SEM (*n = *6) and are representative of three separate experiments. (B) IgG levels in sera collected from mice after oral exposure to* Lactobacillus acidophilus *La 2 (La),* L. casei *ATCC 39539 (Lc),* L. bulgaricus *1489 (NCK 231) (Lb), and* S. thermophilus *St 133 (St) (1.0 × 10⁹ cells/mouse/day) for 7 and 14 days. Data are mean ± SEM (*n = *6) and are representative of two separate experiments.*

ther insight is needed on the influences of various factors of host and microbial origin on the gut microbial ecosystem and potential immune system.

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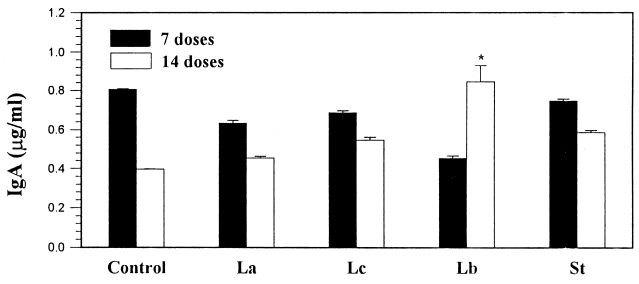

FIGURE 4. *IgA levels in feces collected from mice after oral exposure to* Lactobacillus acidophilus *La 2 (La),* L. casei *ATCC 39539 (Lc),* L. bulgaricus *1489 (NCK 231) (Lb), and* S. thermophilus *St 133 (St) (1.0 × 10⁹ cells/mouse/day) for 7 and 14 days. Data are mean ± SEM (*n = *6) and are representative of two separate experiments.*

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