tenure track position at the Assistant Professor level. The position is a 70% research and 30% teaching appointment in the area of ruminant nutrition. The successful candidate is expected to develop an innovative research program in ruminant nutrition using dairy cattle as the primary animal model and to develop a good working relationship with nutritionists in other departments. Areas of investigation may include, but are not limited to, ruminal function, digestive physiology, nutrient-production interaction, and dairy cattle production practices. The individual selected will be expected to seek extramural funding and to publish research results in refereed and nonrefereed publications. Teaching responsibilities will include an undergraduate course in animal feed formulation, a graduate course in ruminant physiology and metabolism, and assistance with other courses depending on the needs of the department. Advising of undergraduate and graduate students is expected. The individual will be part of a team that is expected to enhance the department's overall efforts in dairy cattle research and teaching. Qualifications include a Ph.D. degree in dairy or animal science or a closely related field with training or interest in dairy cattle nutrition. A research interest in ruminant nutrition is essential. Application deadline is June 1, 1999 or until the position is filled. Applicants should submit a letter describing their research interests and career goals, résumé, and original transcripts and have three letters of reference sent to Bruce F. Jenny, Head, Department of Dairy Science, Louisiana State University, Baton Rouge, LA 70803 (225/388-4411; FAX: 225/388-4008; e-mail: bjenny@agctr.lsu.edu). Louisiana State University is an equal opportunity, affirmative action employer.

• The Department of Animal Science, University of Minnesota, seeks applicants for a 12-month, tenure-track Assistant or Associate Professor position in Livestock Production Systems located in St. Paul. The successful candidate will develop and teach courses in livestock production systems analysis and develop an interdisciplinary research program in livestock production systems analysis. Responsibilities will include teaching undergraduate courses and developing a graduate course in systems principles and methods; developing a comprehensive, interdisciplinary research program utilizing production systems models to identify and evaluate the impact of issues facing the producer, livestock, the community, and the environment; and contribute to outreach educational programs, including interaction with producer, industry, and community groups to identify and prioritize important issues pertaining to livestock production systems. Minimum requirements are a Ph.D. in animal science, agricultural or applied economics, agricultural or biosystems engineering or related fields; demonstrated problem-solving skills utilizing systems methods; strong communication skills; and the ability to work with diverse groups. Preference will be given to individuals with a basic understanding of the economic, social and community, and environmental implications of animal production decisions; evidence of competencies in simulation models, optimization strategies, systems thinking, and holistic problem solving; interdisciplinary research in livestock production; documented teaching experience; and the ability to obtain funding. To apply, provide a letter of application, curriculum vita, all transcripts, and three letters of reference, postmarked by the application deadline of May 15, 1999. Send to: F. Abel Ponce de León, Head, Department of Animal Science, University of Minnesota, 305 Haecker Hall, 1364 Eckles Avenue, St. Paul, MN 55108 (612-624-2722; FAX: 612-625-5789; e-mail: kotva001@tc.umn.edu). For a complete position description, please visit our web page (http://www.animal.agri.umn.edu or http://www.agri.umn.edu/col/dept.html). The University of Minnesota is an equal opportunity educator and employer and specifically invites and encourages applications from women and minorities.

Meetings. . .

May 6–7, 1999 – The 1999 Mid-South Ruminant Nutrition Conference, Sheraton Grand Hotes, Dallas-Fort Worth Airport. For information: Dr. Ellen Jordan, Texas Animal Nutrition Council, Texas A&M at Dallas, 17360 Coit Road, Dallas, Texas 75252 (972/952-9210).

May 12–14, 1999 – Food Irradiation 99. The Solution to the Food Safety Crisis? Washington, DC. For information: Deborah Crommett (207/781-9800; FAX: 208/781-2150;

September 9–10, 1999 – 36th Annual Marschall Cheese Seminar, Santa Clara, CA. For information: Jo Ann Sterenberg, Rhodia, Inc. (219/264-2557; web site: http://www.rhodiadairy.com).

DAIRY FOODS

Ingestion of Yogurt Containing *Lactobacillus acidophilus* and *Bifidobacterium* to Potentiate Immunoglobulin A Responses to Cholera Toxin in Mice

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ABSTRACT

Lactic acid bacteria have been reported to have benefits for the prevention and treatment of some forms of diarrhea and related conditions. To determine whether these effects might involve direct stimulation of the gastrointestinal immune response, we administered yogurt to try to enhance mucosal and systemic antibodies against an orally presented immunogen, cholera toxin. Yogurts were manufactured with starter cultures containing different species and strains of lactic acid bacteria. Mice were fed these yogurts for 3 wk, during which they were also orally immunized twice with 10 μg of cholera toxin. Blood was collected on d 0 and 21, and fecal pellets were collected weekly. Mice that were immunized orally with cholera toxin responded by producing specific intestinal and serum immunoglobulin (Ig)A anti-cholera toxin. Antibody responses of the IgA isotype were significantly increased in mice fed yogurts made with starters containing the conventional yogurt bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* supplemented with *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis*. Yogurt that was manufactured with starters containing only conventional yogurt bacteria produced less IgA anti-cholera toxin than did the control group fed nonfat dry milk. Although strong responses were also observed for IgG anti-cholera toxin in serum, the responses did not differ among groups. Thus, administration of yogurt supplemented with *L. acidophilus* and *Bifidobacterium* spp. enhanced mucosal and systemic IgA responses to the cholera toxin immunogen.

(**Key words**: lactic acid bacteria, cholera toxin, yogurt, immunity)

Abbreviation key: **ABTS** = 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), **CT** = cholera toxin, **IFN** = interferon, **IL** = interleukin, **LPS** = lipopolysaccharide.

INTRODUCTION

Lactic acid bacteria have been documented to have benefits in the prevention and treatment of some forms of diarrhea and related conditions as well as in enhancing immune function (50). The preservation of the integrity of the normal intestinal flora, colonization resistance, adherence, and production of antibacterial substances appear to be important for these effects. Although the precise mechanisms of action are still unclear, yogurt consumption has increased significantly in recent years, presumably in part because of these perceived health benefits (59). Conventional yogurt is a fermented milk produced by the addition of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* to milk. Based on increasing evidence that other lactic acid bacteria such as *Lactobacillus acidophilus* and the bifidobacteria have therapeutic properties, these species have also been added to conventional yogurt or, alternatively, have been used as the main starter culture for the production of new types of yogurt or fermented milks (26, 48). Although conventional yogurt bacteria have a very poor intrinsic resistance to acid and bile (24), *L. acidophilus* and *Bifidobacterium* can tolerate a pH 3 and 2 to 8% concentrations of bile acid (14). Both bacteria can be added in an optimal ratio before inoculation that results in 8×10^8 to 9×10^8 acidophilus cells/ml and 5×10^8 to 8×10^8 bifidobacteria cell/ml (1).

There is evidence (37, 38, 39, 57) that ingestion of lactic acid bacteria exerts an immunomodulatory effect in the gastrointestinal system of both humans and animals. The gastrointestinal system possesses specialized elements that react upon exposure to antigens coming from diet and that result in immune reactions (41). These elements constitute the

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TABLE 1. Composition of lactic acid bacteria in commercial yogurt starter cultures.

Yogurt culture	Lactic acid bacteria	Supplier
Ultra-Gro Direct yogurt culture	*Streptococcus thermophilus* *Lactobacillus delbrueckii* ssp. *bulgaricus*	Sanofi Bio-Industries Waukesha, WI
Sbifidus Direct yogurt culture	*S. thermophilus* *L. delbrueckii* ssp. *bulgaricus* *Lactobacillus acidophilus* *Bifidobacterium* spp.	Sanofi Bio-Industries Waukesha, WI
PY-3 Redi-Set yogurt	*S. thermophilus* *L. delbrueckii* ssp. *bulgaricus* *Lactobacillus acidophilus* *Bifidobacterium bifidum*	Chr. Hansen Inc. Milwaukee, WI
DPL yogurt Quick Start ABY-2C	*S. thermophilus* *Lactobacillus bulgaricus* *L. acidophilus* *Bifidobacterium infantis*	Rhône-Poulenc Madison, WI

mucosal immune system; essentially they are lymphoid tissue containing the full array of immune cells necessary for induction of an immune response, that is, B cells, T cells, macrophages, and accessory cells. The tissues that represent the gut mucosal immunity are Peyer's patches, mesenteric lymph nodes, lamina propria, and intraepithelial lymphocytes (17). The systemic immune compartment is formed by all tissues involved in defense of the internal environment from invading microorganisms and consists of spleen, thymus, bone marrow, and lymph nodes throughout the body. The mucosal and systemic immune compartments can overlap in some of their specific activities. This connection is mediated via circulation of blood and lymph, where B and T cells can migrate from one compartment to the other. The mechanisms by which lactic acid bacteria might activate the mucosal and systemic compartments are still unknown.

One potent antigen that stimulates both the mucosal and systemic compartments is cholera toxin (**CT**) (31). Cholera toxin induces an intestinal secretory IgA response to itself and to additional coadministered antigens (11, 47). A strong local IgA response has been reported in the small intestine as demonstrated by the detection of antibodies against CT in intestinal secretions (55). A strong serum IgG antibody response is also induced by CT immunization, and the cells secreting this isotype appear to be located in Peyer's patches, spleen, and lamina propria. Cholera toxin is known to act as an intestinal adjuvant for many common proteins, viruses, or bacterial polysaccharides and thus mediates a strong IgA memory response (31).

In this study, the effect of yogurt ingestion on the gastrointestinal immune system of the mouse was assessed using CT immunization as a model. Feeding individuals with different types of yogurt made with *L. bulgaricus*, *S. thermophilus* with or without *L. acidophilus*, and *Bifidobacterium* spp. and using CT as an oral protein antigen, we were able to demonstrate the adjuvant activity of yogurt containing *L. acidophilus* and *Bifidobacterium* spp. The results indicated that ingestion of yogurts supplemented with *L. acidophilus* and *Bifidobacterium* spp. enhanced the mucosal and systemic IgA response to CT.

MATERIAL AND METHODS

Yogurt Preparation

Four commercial yogurt starter cultures were obtained from Sanofi Bio-Industries (Waukesha, WI), Chr. Hansen's Laboratories Inc. (Milwaukee, WI), and Rhône-Poulenc, Inc. (Madison, WI). Table 1 summarizes the type of culture present in each starter and its source. The four yogurts were made using pasteurized 12% (wt/vol) NDM (Michigan Milk Producers Association, Ovid, MI) according to the instruction of the culture suppliers. The NDM was inoculated with the starter and mixed. The inoculated mixtures were divided into aliquots in 50-ml sterile conical polyethylene centrifuge tubes and incubated for 6 to 8 h at 37°C to develop a typical yogurt consistency. Yogurts were rapidly cooled and stored at 4°C and were ingested within 21 d. Numbers of total aerobes, streptococci, and bifidobacteria were determined throughout storage.

Enumeration of Yogurt Bacteria

Total aerobic cells, streptococci, and bifidobacteria counts were performed at 0, 1, 2, 3, and 4 wk of storage to determine the viability of the cultures. The MRSL medium [MRS (3) plus 5% (wt/vol) lactose] agar plates, modified *S. thermophilus* agar (Lee's agar) (20), and NPNL (neomycin sulfate, paromomycin sulfate, nalidixic acid, and lithium chloride) agar (58) were used for total aerobic, *S. thermophilus*, and *Bifidobacterium* spp. counts, respectively. After the appropriate dilutions were made, samples were plated using the media mentioned and were incubated for 48 h at 37°C aerobically for total aerobic and *Streptococcus* and anaerobically using anaerobe jars and an anaerobic Gas Pak® (Becton Dickinson Co., Cockeysville, MD) system for *Bifidobacterium*. The colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA.).

Animal Model and Diet

Female B6C3F1 mice (C57BL/6 female × C3H/HeN male), 8 wk old, were obtained from Charles River Laboratories (Raleigh, NC). Ten mice per experimental group were used. Experiments were designed to minimize the numbers of mice required to adequately test the proposed hypothesis, and the experimental protocol was approved by Michigan State University Laboratory Animal Research committee. Mice were held 5 per cage in a windowless room at 25 to 27°C with a light-dark cycle of 12 h of light followed by 12 h of darkness in a negative-pressure ventilated area. They were housed in protected-environment cages (Nalgene, Rochester, NY) that included a transparent polycarbonate body with filter cover and stainless steel wire lid. Water was provided for ad libitum access and was changed every 3 d.

Mice were acclimatized to housing and were fed a nutritionally complete, modified semipurified diet (AIN-93G) as described by the American Institute of Nutrition (46) for at least 1 wk before experiments were started. The study lasted 21 d. Yogurt or NDM control was mixed (1:1, wt/wt) with AIN-93G (ICN Nutritional Biochemical, Cleveland, OH). Yogurt and control diets were prepared fresh and provided daily during the experiment in clean powder feeders with stainless steel grids and lids to reduce spilling. Feeding and treatment schedules during the experimental period are summarized in Figure 1. Weight changes were recorded, and fecal samples were collected weekly. Fecal pellets were immediately processed and were stored at –80°C until analyzed.

Cholera Toxin Immunization

Mice were deprived of food for 2 h before oral immunization. Just before immunization, they were gavaged with 0.5 ml of a solution consisting of 8 parts Hanks balanced salt solution (Sigma Chemical Co., St. Louis, MO) and 2 parts 7.5% sodium bicarbonate to neutralize stomach acidity. Thirty minutes later, 10 μg of CT (Sigma Chemical Co., St. Louis, MO) were administered in 0.25 ml of filter-sterilized PBS. Groups of mice were immunized on d 0 and 14. Five experimental groups were used in the study [control, Ultra-Gro Direct (Sanofi Bio-Industries, Waukesha, WI), Sbifidus Direct (Sanofi Bio-Industries), PY-3 Redi-Set (Chr. Hansen Inc., Milwaukee, WI), and DPL Yogurt Quick Start ABY-2C (Phône-Poulenc, Madison, WI). Food and water were restored immediately after immunization.

Fecal Pellet and Serum Preparation

Fecal samples were prepared as described previously (10). Briefly, feces were collected, aseptically weighed, and placed into centrifuge tubes. Ten milliliters of PBS/g of feces (vol/wt) were added, and the mixture was incubated for 15 min at 25°C. Samples were mixed by vortexing until suspended, left to settle for 15 min, mixed again, and then centrifuged at 22,000 × *g* for 10 min. The supernatant was removed and stored at –80°C for Ig measurement.

Blood was obtained from anesthetized mice from the retroorbital plexus. Serum was obtained after overnight incubation at 4°C and centrifugation at 1000 × *g* for 15 min. Serum samples were divided into aliquots and stored at –80°C prior to monitor response for IgA and IgG anti-CT antibody.

Lymphocyte Culture

Mice were killed 1 wk after the second immunization with CT by cervical dislocation under gentle anesthesia. Spleen and Peyer's patches cultures were chosen to represent systemic and mucosal immune responses, respectively.

Peyer's patches were removed aseptically, placed in a small Petri dish containing 5 ml of RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% (vol/vol) fetal bovine serum (Gibco Laboratories, Chagrin Falls, IL), 2-mercaptoethanol (50 μM), nonessential amino acids (1 mM) (Gibco BRL, Life Technologies, Grand Island, NY), sodium pyruvate (100 mM) (Gibco), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Sigma Chemical Co.) and teased using two sterile cover slides. The cell suspension was passed through

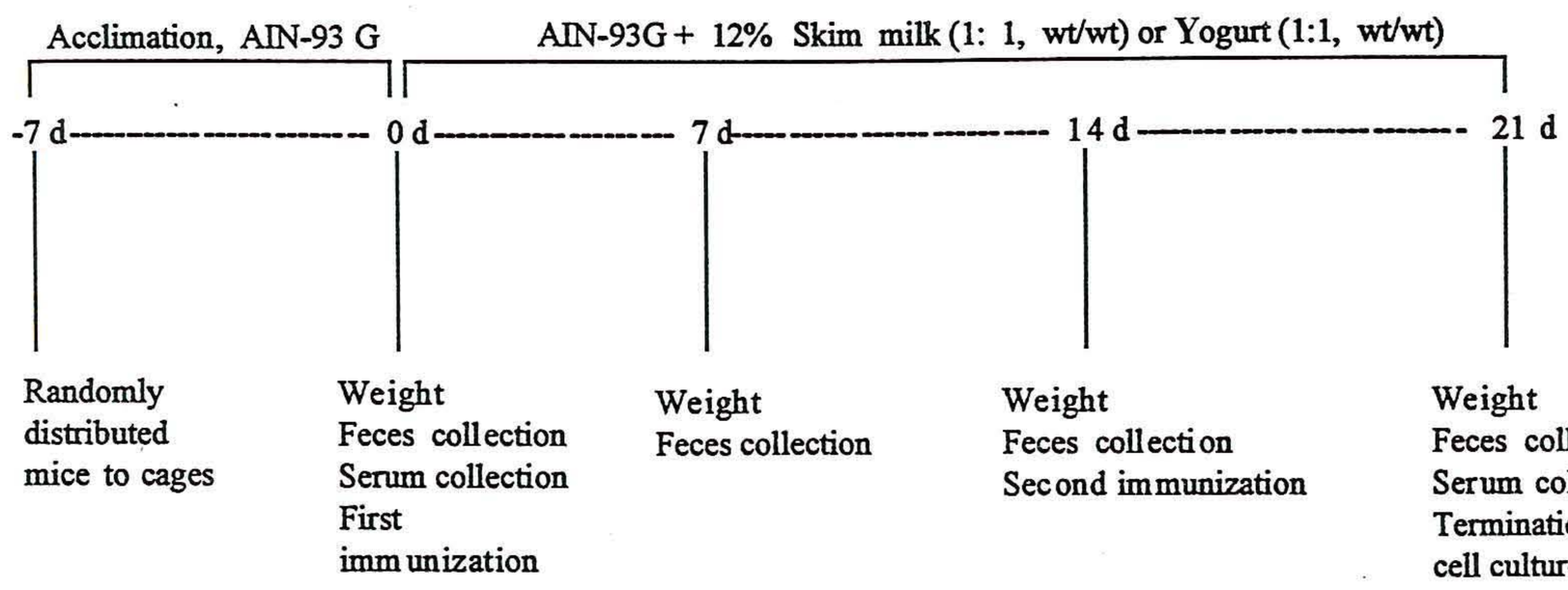

Figure 1. Experimental design for assessing effects of yogurt ingestion on immunoglobulin response to cholera toxin. AIN-93G was from ICN Nutritional Biochemical, Cleveland, OH.

a 70-μm nylon membrane that was fixed on top of a 15-ml sterile centrifuge tube. Five milliliters of fresh RPMI 1640 were added to wash the plate and membrane. Cells were centrifuged at 450 × *g* for 8 to 10 min, resuspended in 2 ml of fresh medium, and counted using 0.4% trypan blue stain (Sigma Chemical Co.) and a hemacytometer. Cell suspensions were kept on ice at all times.

Spleens were removed aseptically, placed in a Petri dish containing 10 ml of RPMI 1460 medium as described, and teased thoroughly; cell suspensions were passed through a sterile 85-mesh screen to remove tissue debris. Lymphocytes were then placed into a 50-ml sterile centrifuge tube, left to settle for 5 min on ice, and then transferred to another sterile tube. The suspension was centrifuged at 450 × *g* for 8 to 10 min, and the supernatant was discarded. Erythrocytes were lysed for 3 min at 25°C in 5 ml of a buffer containing 9 parts 0.16 *M* ammonium chloride plus 1 part 0.17 *M* Tris buffer (pH 7.2). Fresh RPMI 1640 (10 ml) was added, and cells were mixed, centrifuged at 450 × *g* for 10 min, resuspended in 20 ml of fresh medium, and counted. Cells were kept on ice at all times.

Peyer's patch and spleen cells (1×10^5/ml) were cultured in supplemented RPMI 1640 medium in flat-bottomed 48-well (1 ml) tissue culture plates (Fisher Scientific Co., Corning, NY) at 37°C in a 7% CO_2 humidified incubator. Duplicate cultures were stimulated with or without 20 μg/ml of lipopolysaccharide (**LPS**) from *Salmonella typhimurium* (Sigma Chemical Co.). Supernatant was collected at 7 d and stored at –80°C until analysis.

ELISA

Antibody titers in serum, fecal extracts, and cell culture supernatants were determined by an ELISA as described by Jackson et al. (16) with some modifications. Immunolon IV Removawell microtiter strip wells (Dynatech Laboratories Inc., Chantilly, VA) were coated with 100 μl of CT (5 μg/ml) in 0.1 *M* carbonate buffer, pH 9.6. Plates were incubated overnight at 4°C in a humid atmosphere and were washed three times with PBS plus Tween 80 (PBS-Tween). Plates were blocked with 200 μl of 1% BSA in PBS for 1 h at 37°C. The plates were washed again three times with PBS-Tween. Fifty-microliter aliquots of serial dilutions of serum (1:50 to 1:6400, vol/vol), fecal extracts (1:2 to 1:320, vol/vol), or supernatants in 1% BSA-PBS were added in duplicate, and the plates were incubated for 75 min at 37°C. Preimmune serum and fecal extracts at similar dilutions were used on the same plates as controls. Plates were washed three times with PBS-Tween. The secondary antibody consisted of 50 μl/well of horseradish peroxidase conjugated to goat IgG fraction to anti-mouse IgA-α chain (26 μg/ml) or IgG-γ chain (40 μg/ml) (Cappel, ICN Pharmaceuticals, Inc., Aurora, OH) in 1% BSA-PBS. Plates were then incubated at 37°C for 75 min and then washed six times with PBS-Tween. Bound peroxidase was determined by adding 100 μl of 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (**ABTS**) substrate [0.4 m*M* ABTS, 50 m*M* citrate buffer (pH 4.0), and 1.2 m*M* hydrogen peroxide] as described previously by Pestka et al. (42). Absorbance was measured at 405 nm on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). Endpoint titers were expressed as the last dilution that gave an absorbance at 405 nm of ≥0.2 units above the absorbance of preimmune serum and fecal extract.

The CT-specific concentrations of IgA and IgG were quantified in serum, fecal extracts, and cell culture supernatants as described by Pestka et al. (43). Con-

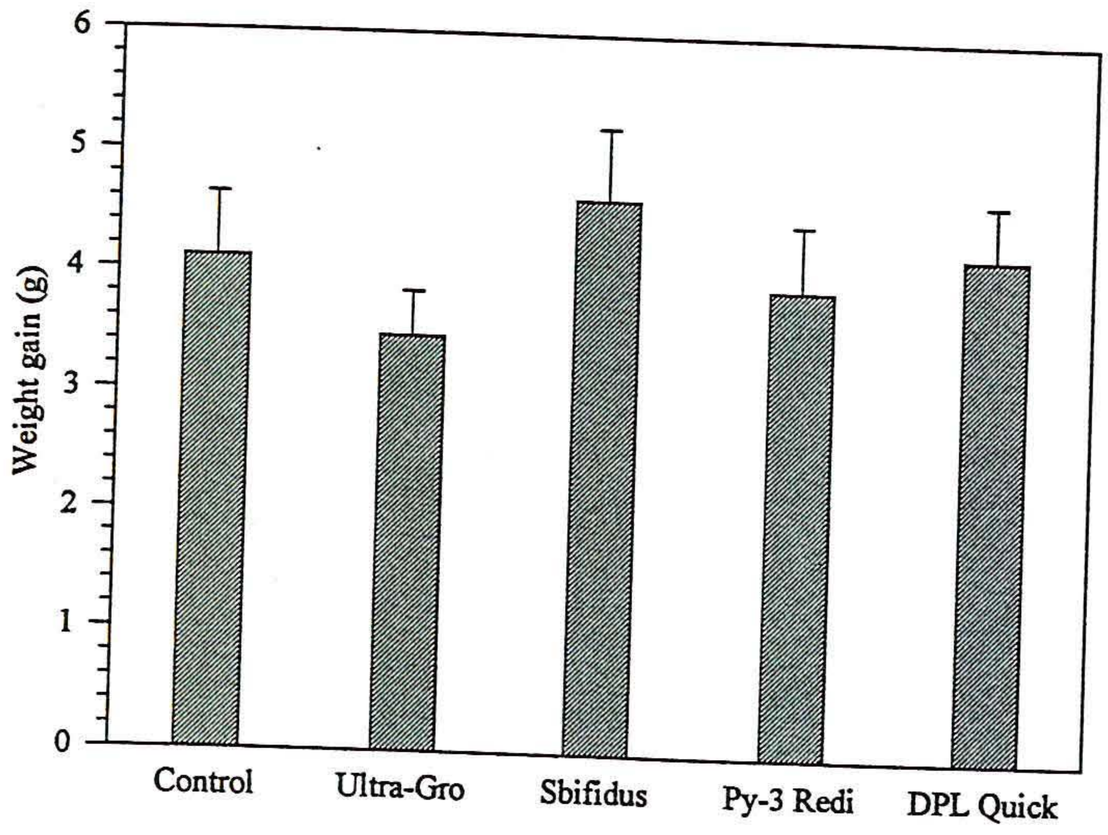

Figure 3. Mean (± SEM) weight gain for mice fed skim milk or yogurt made with lactic acid bacteria and bifidobacteria for 3 wk (n = 10). Mice were weighed at the beginning of the feeding trial and changes in body weight were monitored weekly until the end of the experiment. Yogurts are described in Table 1.

Specific Ig Production

Intestinal and serum anti-CT responses were measured in mice that were immunized orally with 10 μg of CT twice (d 0 and 14) and simultaneously fed with the experimental yogurts. To measure intestinal response, fecal pellets were collected from the various experimental groups, and extracts were tested by ELISA for specific IgA anti-CT (Figure 4). Endpoint titers for CT from all groups of mice before immunization and yogurt feeding were very low (Figure 4A). Anti-CT IgA levels slightly increased 1 wk after mice on control or treatment received the first CT dose, but no significant differences were found among the groups (Figure 4B). Levels appeared to decrease during the 2nd wk for the four treatment groups, and the control group presented a slightly higher level (Figure 4C). One week after a second dose of CT was given, endpoint anti-CT IgA titers differed markedly among all groups (Figure 4D). Based on absorbance measurements, mice fed yogurt made with Sbifidus Direct yogurt culture and PY-3 Redi-Set yogurt culture (containing conventional yogurt bacteria supplemented with *L. acidophilus* and *Bifidobacterium* spp.) exhibited significantly higher titers than did the control group, but the group fed yogurt made with Ultra-Gro Direct yogurt culture (i.e., conventional yogurt bacteria) exhibited a decreased level of specific IgA with respect to the control diet. Gravimetric values for CT-specific IgA were significantly higher for groups fed Sbifidus Direct yogurt culture, PY-3 Redi-Set yogurt culture and DPL yogurt Quick Start ABY-2C than for the control (Table 2). Interestingly, the conventional yogurt (Ultra-Gro Direct) group exhibited significantly lower specific IgA than did the corresponding control group and also with respect to the rest of yogurt treatment groups.

Mice that were immunized orally with CT also responded with specific serum antibody responses of IgA and IgG isotypes. As with coproantibodies, anti-CT IgA concentrations in serum were significantly higher in yogurt treatment groups than in the control group except again for the Ultra-Gro Direct yogurt culture, which lacked *L. acidophilus* and *Bifidobacterium* spp. (Figure 5). As expected, gravimetric estimates of these specific antibodies were higher in serum than in fecal samples (Table 2). Mice also elicited a strong anti-CT IgG response (Figure 6), but titers were lower for IgG than for IgA, and there were no significant differences between the groups of mice fed yogurt or the control diet, except for conventional yogurt culture, which showed significantly less specific IgG than did the control group and other yogurt treatments groups (Table 2). Together these

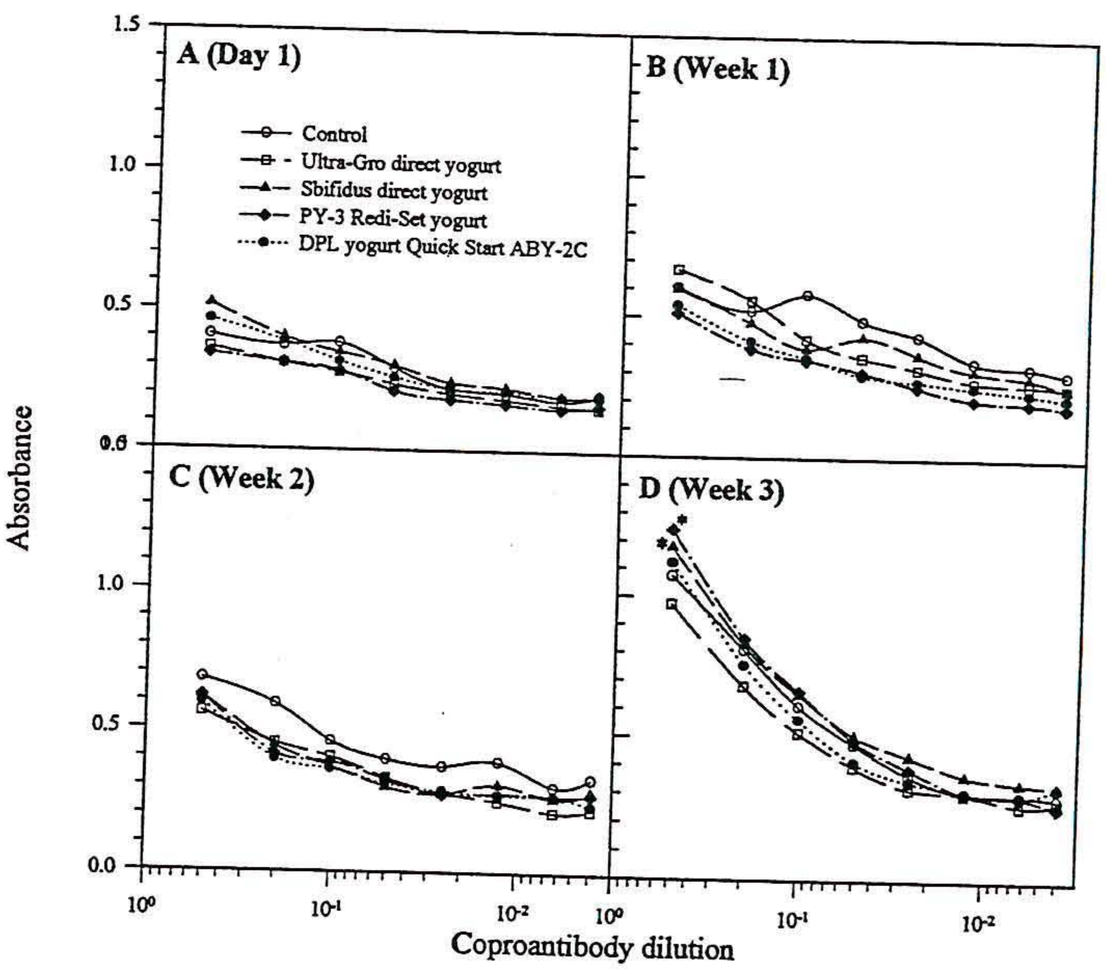

Figure 4. Mean (± SEM) values for specific IgA anti-cholera toxin in fecal samples (coproantibodies). Fresh fecal pellets were collected from each mouse in each group (n = 10; 5 groups) at the times indicated: d 1 of feeding (A), 1 wk after first immunization with cholera toxin and feeding yogurt (B), 2 wk after the first immunization with cholera toxin and feeding yogurt (C), and 1 wk after second immunization with cholera toxin and 3 wk feeding yogurt (D). Extracts were analyzed by ELISA for specific IgA-anti cholera toxin. Asterisk indicates significant difference from the control group ($P \leq 0.05$). Yogurts are described in Table 1.

TABLE 2. Gravimetric estimates of cholera toxin (CT)-specific Ig after 3 wk of oral administration of yogurt.[1]

Yogurt treatment[2]	IgA Anti-CT				IgG Anti-CT	
	Feces[3]		Serum[3]		Serum[3]	
	(μg/g)		(μg/ml)		(μg/ml)	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Control	2.4	0.1	28.8	0.2	1.1	0.1
UltraGro	1.6[ab]	0.0	25.1	1.0	0.4[ab]	0.0
Sbifidus	4.5[a]	0.5	53.3[a]	6.1	0.8	0.2
PY-3 Redi	7.0[a]	0.7	71.8[a]	19.5	1.2	0.2
ABY-2C	3.1[a]	0.8	48.0[a]	2.4	0.8	0.3

[1]Groups of 10 mice were orally immunized with 10 μg of CT on d 1 and 14 of yogurt feeding. Samples of serum and feces were assayed on d 21.

[2]See Table 1 for description of yogurts.

[3]Dilutions used for quantitation of antigen-specific Ig by comparison with a standard reference curve for mouse serum were 1:50 for serum and 1:3 for fecal samples.

[a]Significant ($P < 0.05$) with respect to control treatment (12% skim milk + AIN 93G [1:1]).

[b]Significant ($P < 0.05$) with respect to other yogurt treatments.

results showed that this oral immunization protocol was effective in eliciting CT-antibody responses and suggested that yogurt made with *L. acidophilus* and *Bifidobacterium* spp. enhanced mucosal and systemic IgA responses.

Specific Ig Production in Cell Cultures

The effects of yogurt consumption on Ig production were determined in cultures prepared from Peyer's patches and spleen, which are representative mucosal and systemic lymphoid tissues, respectively. Peyer's patch and spleen cells (1×10^5/ml) were stimulated with or without 20 μg/ml of LPS from *Salmonella typhimurium*, and supernatants were collected and analyzed after 7 d for specific IgA and IgG. Specific IgA concentrations were similar in Peyer's patch cell cultures for all treatments with respect to the control. A trend toward increased IgA anti-CT levels was observed in spleen cell cultures stimulated with LPS from groups treated with yogurt compared with levels in the control group, but these effects were not significant (data not shown). Similar results were obtained when we analyzed for CT-specific IgG.

DISCUSSION

This study demonstrated that administration of yogurt differentially affected mucosal and systemic IgA responses to CT in vivo. Specifically, yogurt starter cultures containing *L. acidophilus* and any type of *Bifidobacterium* spp. had an effect on mice that was reflected by specific IgA anti-CT production compared with that of conventional yogurt starter cultures containing only *L. bulgaricus* and *S. thermophilus*. Thus, the bacteria present in the starter culture seemed to be critically important for the humoral immune response studied here, which suggests that several factors specifically related to a bacterial species might affect the extent to which yogurt alters immune function.

To exert a maximal influence on gastrointestinal function and to be able to act as probiotics, lactic acid bacteria may need to be present in high number in fermented milks and to survive the digestive process

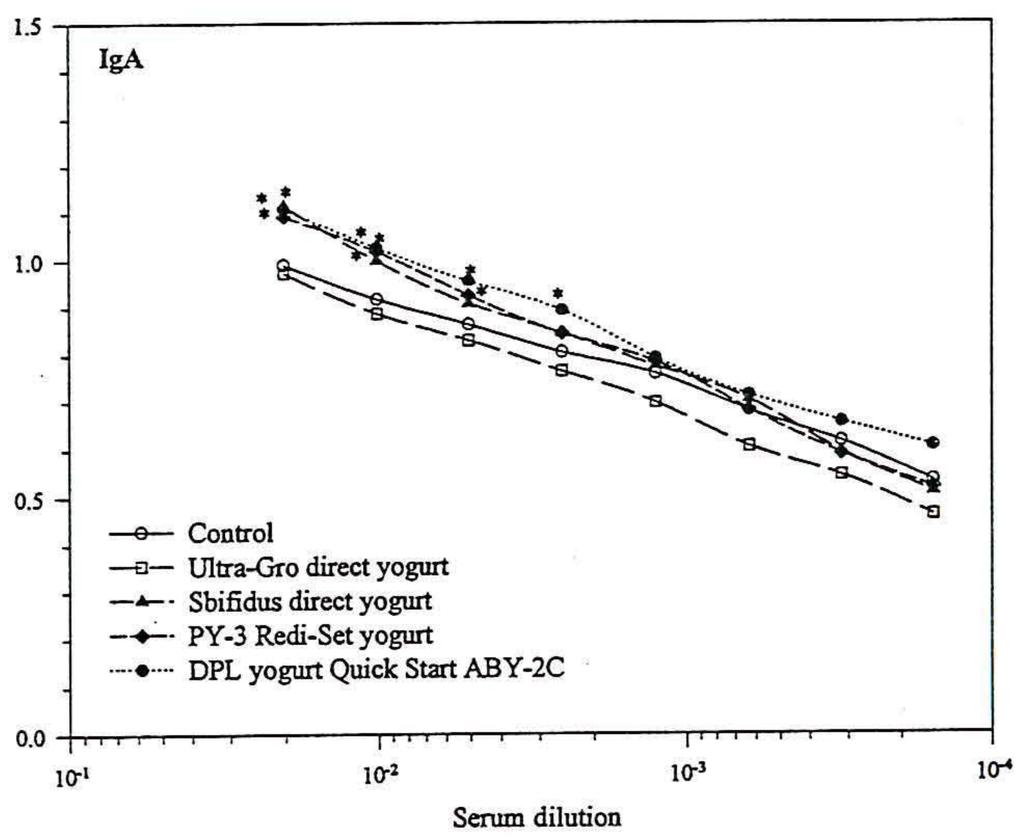

Figure 5. Mean values for specific IgA anti-cholera toxin in serum samples. Serum samples were collected 1 wk after final immunization and analyzed by ELISA for specific IgA anti-cholera toxin. Preimmune serum absorbance = 0.12. Asterisk indicates significant difference from the control group ($P \leq 0.05$). Yogurts are described in Table 1.

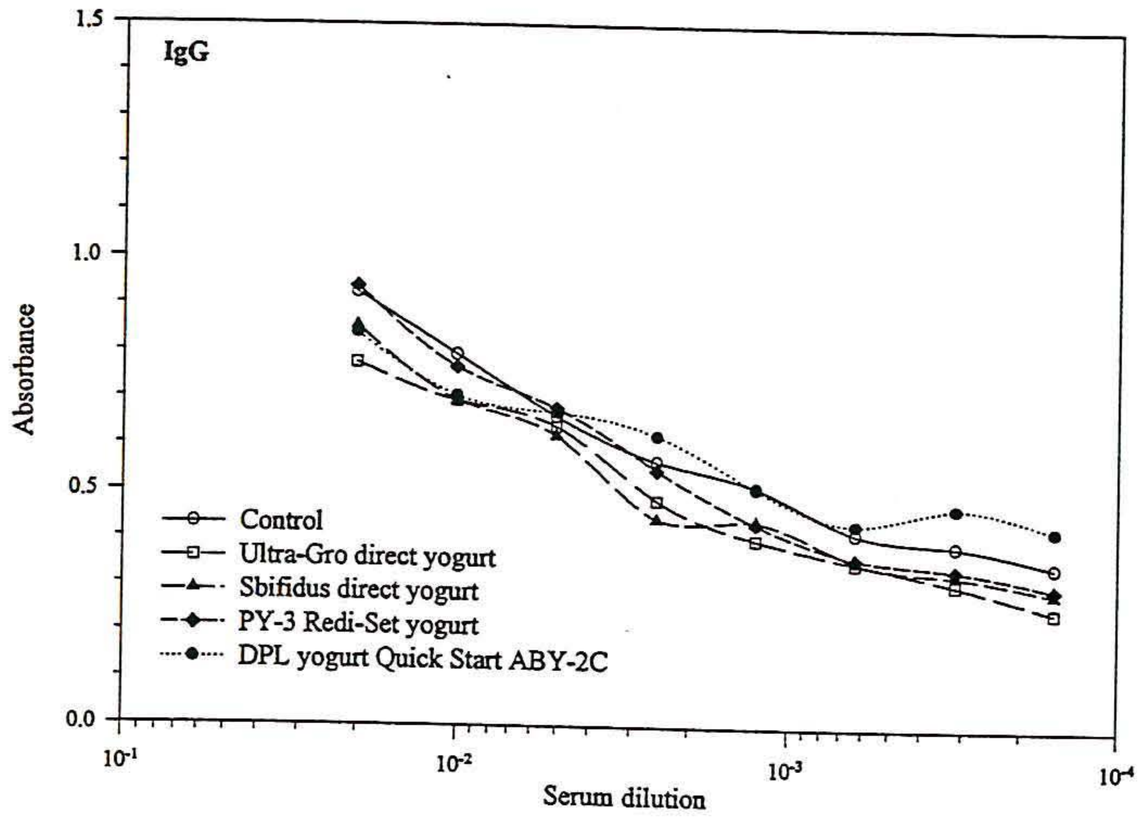

Figure 6. Mean (± SEM) values for specific IgG anti-cholera toxin in serum samples. Serum samples were collected 1 wk after final immunization and analyzed by ELISA for specific IgG anti-cholera toxin. Preimmune serum absorbance = 0.10. Asterisk indicates significant difference from the control group ($P \leq 0.05$). Yogurts are described in Table 1.

(18). In this experiment, the viability of bacteria in all yogurts remained high and within the normal values for commercial fermented milks (53). This result is important because the ability of *L. bulgaricus* and *S. thermophilus* to persist in the gut after administration in mice is questionable and because there is a general consensus that continuous ingestion is needed to maintain colonization in animal models (13). The capacity to tolerate low pH and high bile concentrations is advantageous for the survival of *L. acidophilus* and *Bifidobacterium bifidum* inside the gut (1). It is possible that starter cultures containing *L. acidophilus* and *Bifidobacterium* spp. were able to persist in the mouse gut; the classic yogurt bacteria, *L. bulgaricus* and *S. thermophilus*, were washed out faster.

B6C3F1 mice were chosen for this study because of increased hardiness and longevity characteristics of heterosis and because the wider genetic diversity is more characteristic of human populations (12). This hardiness is especially critical for conducting feeding studies, and the use of genetically identical mice minimizes variability encountered in immunological experiments. No diarrhea, food refusal, or discomfort was observed at any time during the feeding trial. We have previously shown that the mean feed intake for yogurt produced with these same starters ranged from 5.4 to 5.8 g/d per mouse (C. Ha and J. Pestka, 1999, unpublished data).

The mice fed NDM or yogurt were provided with a semipurified powder diet (AIN-93G) at a 1:1 (wt/wt) ratio, and the gain in body weight was not significantly different between control and treatment groups. Puri et al. (45) have similarly found no difference in the growth rate of mice fed yogurt containing the classic cultures (*L. bulgaricus* and *S. thermophilus*) or milk for 4 wk. In this latter study, the yogurt diet was also prepared by mixing yogurt with powdered pellets at a 1:1 ratio. In contrast to these latter findings, better growth was observed in rats fed freeze-dried yogurt (15) and classic yogurt (25) than in rats fed milk. This discrepancy among studies might be explained by the different animal model employed and because the study presented here used yogurt mixed with a powdered food, but some others (15, 25) fed only unsupplemented yogurt or milk.

It should be noted that mice orally immunized three times at weekly intervals with 10 μg of CT produced maximum IgA anti-CT responses in fecal samples and IgG anti-CT in serum on d 21 (60). We reduced immunization frequency, knowing the capacity of CT to act as a potent mucosal antigen, and administered just two doses because our goal was not to achieve a maximal antibody response to CT but rather to determine whether a yogurt treatment enhanced the response.

The observation that fecal and serum IgA anti-CT levels were higher in mice fed yogurts with bifidobacteria and *L. acidophilus* than in the mice fed the control yogurt is likely to be related to enhanced stimulation of the gut mucosa immune system. In support of this contention, we found that feeding mice with *L. casei* increases IgA to enteropathogens (35) and induces a protective effect against *Escherichia coli*, *Listeria monocytogenes* (29, 30), and *Mycobacterium bovis* (49). Other studies have reported that feeding mice fermented milks containing *L. casei* (33), *L. acidophilus*, and yogurt cultures (*L. bulgaricus* and *S. thermophilus*) (36) exerts a protective effect against intestinal pathogens by activating lymphoid follicles and increasing production of immunoglobulins (2). Also, serum IgA concentrations after challenge with *Salmonella* spp. were significantly higher in mice fed classic yogurt than in mice fed milk (45). Portier et al. (44) prepared sera from mice fed yogurt fermented by *L. bulgaricus* and *S. thermophilus*, the identical but heated product, or a *L. casei* fermented milk and then vaccinated with partially purified CT (three times intraperitoneally [40, 100, and 200 μg per mouse] and once orally [100 μg per mouse]) at weekly intervals. When analyzed by a vibriocidal test for specific antibodies against two highly correlated serotypes (Ogawa strain and Inaba

strain) of *Vibrio cholerae*, significant differences ($P > 0.05$) to the Ogawa serotype but not to the Inaba serotype were found. Thus, in some case, it seems that classic yogurt has adjuvant properties and the capacity to stimulate the systemic immune system, which contrasts with our findings that yogurt made with *L. bulgaricus* and *S. thermophilus* had no effect on anti-CT IgA at the systemic level. This contradiction could be attributed to the different extract of toxin used, the different dose or immunization protocol followed, and the different assay used to measure specific antibodies.

The mechanisms by which yogurt made with *Bifidobacterium* spp. or *L. acidophilus* stimulate the gut immune system are unclear. The mucosal immune system has B lymphoblasts derived from the gut-associated lymphoid tissue (41). The Peyer's patches are the central focus for the induction of T- and B-cell responses following an oral immunization. These organs lie below a specialized layer of epithelial cells called M cells. Once the antigen has traversed the M cells, antigen-presenting cells within the Peyer's patch can take up the antigen and present it to nearby T cells. There are two different clones of T cells: T-helper cells can be classified as Th1 [producing interleukins (**IL**)-2, IL-3 and interferon (**IFN**)-γ] and Th2 (producing IL-6, IL-4, IL-5, IL-10) (32). Some of these cytokines are able to activate B cells and mediate proliferation, switching, and differentiation of these cells to become committed to secrete IgA (52). The B cells within the follicles expressing IgM or IgD on their surface upon stimulation can proliferate and differentiate into lymphoblasts expressing IgA on their surface. The B-cell lymphocytes pass into the efferent lymphatics to the mesenteric lymph nodes and from there can enter the systemic circulation via the thoracic duct and enter distant effector sites as lamina propria of the intestine. At these effector sites, B cells proliferate and mature into IgA plasma cells in response to certain signaling by cytokines produced by T cells and macrophages. Plasma cells produce polymeric IgA that is then secreted across the epithelial cell into the lumen (41). The IgA that is secreted by B cells in the lamina propria can be released to the intestine following the bowel content or to the general circulation, raising the serum IgA concentrations.

The CT is composed of subunit A (posttranslationally cleaved into toxigenic A1 and A2 peptides) and subunit B. Subunit B is a homopentamer that serves as a carrier for the subunit A by binding to monosialoganglioside GM1 present in the intestinal cells. A conformational change in this GM1 allows the A1 peptide to penetrate the cell. The mechanism is unknown but could involve some type of endocytosis (31) or direct translocation of the A1 component through the lipid bilayer. Internalized CT drives B cells differentiation to IgA-committed precursors. The CT might affect Peyer's patch B cells either directly by contact or indirectly by activation of macrophages and T cells.

One possible mechanism by which certain yogurts alter the immune response on gut-associated lymphoid tissue may involve the generation of a local signal at the intestinal mucosal surface or by improved translocation of antigen through the mucosal barrier. Some bacterial components with immunomodulatory activities seem to be lipoteichoic acids, endotoxic LPS, and peptidoglycans, which are species and strain specific. Related to these bacterial fractions, other studies (21, 22, 23) conducted by this laboratory showed that whole, nonviable lactic acid bacteria cells and their cell-wall and cytoplasmic fractions stimulated macrophages in vitro to release cytokines and nitric oxide (M. Tejada-Simon and J. Pestka, 1999, unpublished data).

Lactic acid bacteria may activate macrophages directly. Administration of fermented milks with lactic acid bacteria (*L. bulgaricus* and *S. thermophilus*, *L. casei*, *L. acidophilus*, and *Bifidobacterium* spp.) apparently enhances immune response in animal studies by activating macrophages and lymphocytes (40). Consistent with our data, it has been observed that classic yogurt induced less phagocytic activity in macrophages than did *L. acidophilus* and *Bifidobacterium* fermented milks (27, 28). Oral administration of *L. acidophilus* strain La1 and *B. bifidum* strain Bb12 at doses of 7×10^{10} cfu/d (51) to humans increases phagocytic activity in blood. Activated macrophages were important in the resistance of the host to infections and tumors (37, 38, 39). If a mixture of *L. casei* and *L. acidophilus* is administered orally, increased lymphocytic activity and in vitro peritoneal macrophage phagocytic activity are found (37). Macrophages and lymphocytes are also activated with administration of *L. acidophilus* and *S. thermophilus* (39). When administered to animals in a yogurt form, these bacteria seem to increase the number of spleen germinal centers and T and B lymphocytes and to decrease preexisting enterobacterial infections (4, 5, 6, 8). In humans fed yogurt, research (7, 8, 9) showed an increase of B and NK cells in lymph nodes and IFN-γ production.

Administration of lactic acid bacteria or their fermented milks have also been reported to affect the production of cytokines. The administration of 1×10^8

cfu/d for 15 d of *L. bulgaricus* and *S. thermophilus* used in the manufacture of fermented milk products, either in mice (56) or humans (57), resulted in increased concentrations of IL-1β, tumor necrosis factor-α, IFN-γ. This effect was even higher with milks fermented with *L. casei*, *L. acidophilus*, and *Bifidobacterium* spp. (57).

Gangliosides are glycosphingolipids that contain sialic acid and neuraminic acid (important component of bacterial cell walls) (34). They are found in apical membranes of all intestinal epithelial cells. Most pathogens, including *Helicobacter pylori*, are able to attach themselves to epithelial cell surfaces through specific structures namely GM3 gangliosides, and to damage the mucus coat by protease activity (54). *Lactobacillus casei* was also found to bind in the intestinal tract to some specific glycosphingolipids possessing short sugar chains and galactosyl moiety (61). Present also in milk and other dairy products, gangliosides inhibit enterotoxin activity from *V. cholerae* and *Escherichia coli* in vitro and in vivo in human studies (19). Yogurts might present differential amounts of these gangliosides, which may potentiate or attenuate immunogenicity of toxins like CT.

CONCLUSIONS

In summary, a murine model has been established in which the adjuvant activity of yogurt containing *L. acidophilus* and *Bifidobacterium* spp. was demonstrated by generating a strong gut mucosa and systemic IgA anti-CT response. Yogurt manufactured with starters containing only yogurt bacteria *L. bulgaricus* and *S. thermophilus* produced decreased IgA-anti CT when compared with either the control group fed the NDM or other groups fed different types of yogurt made with *L. bulgaricus* and *S. thermophilus* supplemented with *L. acidophilus* and *Bifidobacterium* spp. Further investigation of how these lactic cultures modulate the IgA response is warranted.

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Effect of Fat Reduction on Chemical Composition, Proteolysis, Functionality, and Yield of Mozzarella Cheese[1]

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ABSTRACT

Mozzarella cheese was made from skim milk standardized with cream (unhomogenized, 40% milk fat) to achieve four different target fat percentages in the cheese (ca. 5, 10, 15, and 25%). No statistically significant differences were detected for cheese manufacturing time, stretching time, concentration of salt in the moisture phase, pH, or calcium as a percentage of the protein in the cheese between treatments. As the fat percentage was reduced, there was an increase in the moisture and protein content of the cheese. However, because the moisture did not replace the fat on an equal basis, there was a significant decrease in the moisture in the nonfat substance in the cheese as the fat percentage was reduced. This decrease in total filler volume (fat plus moisture) was associated with an increase in the hardness of the unmelted cheese. Whiteness and opacity of the unmelted cheese decreased as the fat content decreased. Pizza baking performance, meltability, and free oil release significantly decreased as the fat percentage decreased. The minimum amount of free oil release necessary to obtain proper functionality during pizza baking was between 0.22 and 2.52 g of fat/100 g of cheese. Actual cheese yield was about 30% lower for cheese containing 5% fat than for cheese with 25% fat. Maximizing fat recovery in the cheese becomes less important to maintain high cheese yield, and moisture control and the retention of solids in the water phase become more important as the fat content of the cheese is reduced.

(**Key words**: fat reduction, Mozzarella cheese, composition, functionality)

Abbreviation key: **AV** = apparent viscosity, **FO** = free oil, **LMPS** = low moisture part skim, **MNFS** = moisture in the nonfat substance, **TPA** = texture profile analysis.

INTRODUCTION

Reduction of fat intake in the American diet is recommended by the US Department of Agriculture (32). Fat reduction in the diet is important based on the scientific evidence linking diets high in fat to coronary heart disease and certain types of cancer (36). To help consumers achieve healthier eating, the food industry has responded by developing reduced fat foods. In 1994, the cheese industry introduced 247 new cheese products, many of which were reduced fat cheeses (8, 22).

Driven by the popularity of pizza, Mozzarella cheese sales continue to grow. Low moisture part-skim (**LMPS**) Mozzarella cheese is commonly used for pizza because of its desirable functionality (13). However, LMPS Mozzarella cheese, by definition, contains 14 to 22% fat, depending on its moisture content (9). Given the healthier eating goals of consumers and the continued demand for pizza, there has been an interest in developing a lower fat Mozzarella cheese (10, 20, 29, 30, 31).

Fat performs many important functions within a food. For cheese, fat contributes to the taste, texture, functionality, and appearance. Because about 75% of all Mozzarella cheese is used as an ingredient for pizza, proper melt (shred fusion) and appearance (browning and blistering upon heating) are important characteristics (1, 12). When fat is removed from cheese, the overall quality of the cheese decreases (10, 20, 29, 30, 31).

Tunick et al. (31) used homogenization of milk and a lower cooking temperature as a means to enhance the functionality of reduced fat Mozzarella cheese. Those researchers found that homogenization resulted in a reduced fat Mozzarella cheese (ca. 10%

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