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Research Article

Probiotic bacteria are antagonistic to *Salmonella enterica* and *Campylobacter jejuni* and influence host lymphocyte responses in human microbiota-associated immunodeficient and immunocompetent mice

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A defined human microbiota-associated (HMA) mouse model in BALB/c and immunodeficient Tgε26 mice was used to assess the ability of probiotic lactobacilli and bifidobacteria to enhance colonization resistance to gastrointestinal (GI) tract pathogens. Probiotic bacteria (1×10^8 colony forming unit (CFU)/mL) successfully excluded *Campylobacter jejuni* from both strains of mice 7 days after challenge. The probiotic bacteria also reduced the number of *Salmonella* in the large intestines of both mouse strains. The nylon wool fractionated spleen lymphocyte populations were incubated with *Salmonella* or *C. jejuni* antigens. The probiotic treatments did not affect lymphocyte proliferation to *C. jejuni* antigens, but significantly increased proliferation of lymphocytes to *Salmonella* antigens by 68 and 55%, respectively, over untreated mice. Caspase 3/7 activation was significantly reduced 33 and 38% in the T and B lymphocyte fractions, respectively, of probiotic-treated, *Salmonella*-challenged HMA BALB/c mice, suggesting that lymphocyte rescue from apoptosis was occurring as a result of probiotic bacteria activity. These results revealed an immunosuppressive activity by *Salmonella* that was inhibited by the presence of probiotic bacteria. In summary, lactobacilli and bifidobacteria competitively excluded *C. jejuni* from immunocompetent and immunodeficient mice and antagonized an observable *Salmonella*-induced immunosuppression in immunocompetent mice.

Keywords: Food safety / Germfree animals / Intestinal bacteria / Immunodeficiency / Probiotics

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1 Introduction

The intestinal microbiota is the group of commensal microorganisms living in the gastrointestinal (GI) tract that provides a barrier to invasion by pathogenic organisms such as *Salmonella enterica* and *Campylobacter jejuni* by a process called colonization resistance. This process occurs by several mechanisms, including competition for nutrients and attachment sites [1, 2] and the production of inhibitory substances like toxins, bacteriocins, and SCFAs [3–6]. Together, these mechanisms aid host defenses in resistance to food-borne illness. Live microbial food ingredients, or probiotics, are consumed with the intent of achieving a nutritional or health benefit additional to the beneficial effects of the indigenous microbiota. One such benefit is enhancement of microbiota colonization resistance against infection by intestinal pathogens. Several human and animal studies provide limited, but compelling evidence that probiotic bacteria are effective preventative treatments for some of these infectious diseases [7–10].

Drugs, dietary changes, and environmental factors may affect the integrity of the GI tract microbiota and its ability to compete against food-borne pathogens [11]. The effects of probiotic supplements on the microbial ecology of the complex GI tract microbiota are not well understood. The potential for probiotic-induced changes in the composition of the GI tract microbiota to harm the human consumer is currently unclear, but one probiotic trial in patients with

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Abbreviations: BBE, bacteroides bile esculin; **bifido,** Bifidobacterium agar; **CFU,** colony forming units; **GI,** gastrointestinal; **GF,** germfree; **HMA,** human microbiota-associated; **MRS,** deMann, Rogosa, Sharpe

severe acute pancreatitis has recently revealed the potential for lethal effects by the treatment organisms [12]. A model system that incorporates a simple defined human microbiota with sufficient microbial diversity to provide competitive exclusion and incorporates it into an immunodeficient animal host could be very useful to study the effects of probiotics on enteric infections. Human microbiota-associated (HMA) mice have been used successfully as models of intestinal microbial metabolism [13]. Previous models consisted of mice that were inoculated with human fecal bacteria, but not the populations of bacteria associated with intestinal mucosae. These latter studies reported that some, but not all of the ex-germ-free mice treated with human fecal bacteria demonstrated metabolic activities similar to those measured in human fecal bacteria [14]. It is well known; however, that the fecal bacteria composition is different from the composition of the bacteria associated with the colonic mucosa [15–17] or the mucosal surface of the small intestine [18, 19]. In the present study, a gnotobiotic mouse model [20] was developed with a defined intestinal microbiota similar to those found in humans to evaluate the safety, efficacy, and mechanisms of action of probiotic bacteria in immunodeficient and immunocompetent hosts.

Although many probiotic bacteria are generally recognized as safe because of their long history of use in the fermented foods industry, that status of safety is known mainly in immunocompetent hosts [21]. Large numbers of immunodeficient individuals are currently found in the world today due to medical advances in chemotherapy, organ transplantation, and the recent growth of human immunodeficiency virus infections. Little data is currently available on the risks of probiotics in these people. An *in vivo* experimental system has significant advantages over *in vitro* systems, because microenvironments within the intestinal ecosystem vary over the length of the intestinal tract for such parameters as acidity, oxygen tension, and nutrient availability, which are much more difficult to control *in vitro* [22]. Thus, an *in vivo* model system was considered for the present study. The Tgε26 mouse strain is immunodeficient because it has a human CD3ε gene insertion that inhibits expression of either the murine or human epsilon gene, which prevents the maturation of T cells [23]. It was expected to have similar immune response limitations to the *bg/bg-nu/nu* immunodeficient mouse strain with which we had used previously to study probiotic protection against *Candida albicans* infections [21, 24–26]. In the present study, the Tgε26 mouse strain was used to evaluate whether the probiotic bacteria would cause any illness and whether they could prevent colonization of the mice by enteric pathogenic bacteria.

Probiotics have potential not only to provide colonization resistance but also immune system stimulation for the immunodeficient host [21, 25]. Results of studies with severely immunodeficient mice have shown that some strains of probiotic bacteria are safe [21, 26], and some probiotic bacteria can improve the immune functions of the host [25]. A possible mechanism for probiotic enhancement of colonization resistance is through stimulation or suppression of the host immune responses to enteric pathogenic bacteria. The immune system effects of probiotics include: development of immunological tolerance to food antigens, enhanced production of secretory IgA, induction of M-cell development in Peyer's patches, nonspecific immunostimulation, and enhanced antigen-specific antibody production [27]. In a gnotobiotic mouse model of candidiasis, antigen specific and nonspecific probiotic immune enhancements were observed in immunodeficient animals [24]. If probiotics can provide enhancement of host defenses against *C. albicans* infections, perhaps they can be effective in bacterial infections. Enhancement of host defenses against enteric infections by *S. enterica* would be very beneficial, especially if the probiotic effect could overcome the immune system-inhibiting effects of salmonellosis. Phagocytic cells that encounter *S. enterica* serotype Typhimurium are induced into programed cell death by activation of caspase 1 by the Sip B protein produced by the bacteria [28]. However, less is known about the T and B cells that respond to *Salmonella* infections than the phagocytic cells.

It has long been understood that activated lymphocytes tend to move rapidly into a state of programmed cell death if they are not presented with signals that block progression of apoptosis [29]. Persistence of activated lymphocytes by inhibition of apoptosis could be a mechanism for enhancement of an immune response to an active infection. Our hypothesis in the present study is that probiotic bacteria can enhance primary host responses to oral *S. enterica* and *C. jejuni* challenges by activating lymphocyte proliferation and preventing clonal deletion by apoptosis of lymphocytes reactive to the pathogen. In order to observe the induction of lymphocyte persistence by probiotics, we tested splenic lymphocytes from probiotic treated and untreated mice after oral challenge with *S. enterica* or *C. jejuni* for lymphoproliferation and for activation of the penultimate caspases 3 and 7 in the caspase apoptosis activation pathway.

2 Materials and methods

2.1 Experimental design

Germfree (GF) BALB/c and immunodeficient Tgε26 mice were orally inoculated with a defined mixture of human GI tract bacteria to generate HMA mice. After 14 days, the HMA mice were moved to other sterile isolators, one containing 18 HMA mice of each strain, and, the other contained 12 mice of each strain that were orally inoculated with probiotic bacteria, as previously described [24]. Seven days later, the HMA mice and the probiotic-fed HMA mice were moved to other isolators for oral challenge with the human enteric pathogens *Salmonella enterica* or *C. jejuni.* The mice were removed from the isolators at 1 wk after

Table 1. Components of the model human microbiota (ATCC no.)

Group 1[a)]	Group 2	Group 3
Escherichia coli (25922)[b, c)]	*Kluyvera ascorbata* (14236)[b, c)]	*Collinsella aerofaciens* (25986)[b)]
Enterococcus durans (6056)[d)]	*Propionibacterium granulosum* (11829)[c)]	*Eubacterium biforme* (27806)[b)]
E. faecalis (27274)[b, d)]	*P. granulosum* (25564)[c)]	*Fusobacterium nucleatum* (25586)[b)]
Bacteroides fragilis (25285)[b, d)]	*L. ruminus* (25644)[b, d)]	*Anaerococcus hydrogenalis* (49630)[b)]
B. thetaiotaomicron (29148)[b, d)]	*L. salivarius* (11741)[d)]	*Ruminococcus bromii* (27255)
E. faecium (6569)[b, d)]	*L. johnsonii* (33200)[d)]	*Eubacterium hadrum* (29173)
Lactococcus lactis (11454)[c)]	*L. reuteri* (23272)[d)]	*R. productus* (27340)[b)]
B. vulgatus (8482)[b, d)]	*B. longum* (15707)[d)]	*R. obeum* (29174)[b)]
B. distasonis (8503)[b, d)]	*B. adolescentis* (15703)[b, d)]	*Clostridium leptum* (29065)[b)]
Pediococcus acidilactici (8042)[c)]	*B. bifidum* (29521)[d)]	
R. infantarius (BAA-103)[d)]	*Morganella morganii* (25830)[c)]	
	Enterobacter aerogenes (29940)[b, d)]	
	Providencia alcalifaciens (9886)[b, c)]	

a) Three bacteria mixtures were prepared and mice were orally inoculated with each of the three groups over successive days.
b) Species that were isolated from pooled cecum samples obtained from six HMA BALB/c mice 14 days after the start of microbial succession and subsequently identified using cellular fatty acid methyl ester composition (Microbial ID) and MicroSeq 16S rRNA sequence homology assays (Applied Biosystems).
c) Relative abundances of strains in the final mixture is 8 $\log_{10}$.
d) And 10 $\log_{10}$.

challenge, and intestinal tissues were removed for assays. Small and large intestines, with their contents, were separately homogenized at 4°C with a Tissue–Tearor homogenizer (BioSpec Products, Bartlesville, OK) on a setting of no. 2 for 15 s bursts and the homogenates were quantitatively cultured for the challenge pathogens (*Salmonella* or *C. jejuni*), representative species groups of the model human microbiota, and the test probiotic mixture.

Spleens were excised from the mice, minced through 100 μm nylon mesh cell strainers (BD Biosciences, Bedford, MA) and the cells were enriched on nylon wool columns (Polysciences, Warrington, PA) for T cell and B cell fractions. The nylon nonadherent fraction of spleen cells is referred to as a T cell fraction in this manuscript for purposes of comparison to BALB/c mice, but the Tgε26 mice do not have mature T cells in this fraction [23].

2.2 Model human GI tract microbiota

The composition of the bacterial mixture used to colonize the mice (Table 1) reflects what is known about the compositions of the microbiota associated with the human small and large intestinal mucosae [10, 16–18, 20, 30–32]. Since some bacteria in the model human microbiota were obligate anaerobes, their short-term aerobic tolerance was tested by overnight anaerobic culture of each kind of bacteria after one hour of aerobic incubation at 37°C in a humidified atmosphere of 95% air, 5% CO_2. The bacteria species listed in Table 1 were cultured in their preferred media (as recommended by the American Type Culture Collection (ATCC, Rockville, MD) under anaerobic conditions. Each culture was centrifuged at 10 000 × *g* for 10 min, then the supernatants were decanted, and the bacteria pellets were suspended in sterile milk stabilizer (8% powdered skim milk, 5% sucrose, and 0.5% thiourea in water). Samples of each bacteria culture were serially diluted and enumerated by agar plate dilution analyses on Brucella Blood Agar with hemin and vitamin-K (REMEL Laboratories, Lenexa, KS) incubated at 37°C in a hydrogen-free anaerobic atmosphere using ascorbic acid sachets (REMEL Laboratories). The bacteria were mixed without further culture to maintain the relative abundance of each isolate in the model human microbiota. Relative abundances of each strain in the final mixture [10, 16–18] are indicated in Table 1.

The capacity of the model microbiota and the individual bacterial mixtures used for succession colonization of the mice were tested with an *in vitro* assay for their efficacy to promote colonization resistance against *Salmonella* [33]. Reduction in the numbers of *Salmonella* invading Caco-2 enterocyte (human colonic adenocarcinoma cell line) culture cells by the bacteria mixtures were compared to a media control and also to a competitive exclusion product that has proven efficacy against *Salmonella* colonization of poultry *in vivo* [34].

2.3 Probiotic bacteria

Samples of probiotic products containing *Lactobacillus* sp. and *Bifidobacterium* sp. for human consumption were obtained from a health food store. The bacteria, isolated and characterized from the powdered products (intended to be mixed with infant formula), were cultured for isolation of component bacteria on deMann, Rogosa, Sharpe (MRS) agar (REMEL Laboratories) and *Bifidobacterium* agars (Bifido) (Anaerobe Systems, Morgan Hill, CA) incubated in an anaerobic chamber (Coy Laboratories, Grass Mill,

MI) with an atmosphere of 90% N_2, 5% CO_2, 5% H_2 (Airgas, Pine Bluff, AR) for 48 h at 37°C. Probiotic powder was suspended in MRS broth and streaked for isolation on MRS and Bifido selective media plates. Thirty colonies from each plate were isolated and observed with Gram stains. Six colonies of each morphologically different type from each medium were identified with cellular fatty acid methyl ester composition (Microbial ID, Newark, DE) and MicroSeq 16S rRNA sequence homology assays (Applied Biosystems, Foster City, CA). Serial dilution plate counts on MRS or Bifido agar were used to quantify the components of the probiotic products. Probiotic products were administered to the mice suspended in their drinking water at a concentration of 8.0 $\log_{10}$ colony forming unit (CFU)/mL.

2.4 Culture of challenge bacteria

The challenge organisms used in this study, *S. enterica* and *C. jejuni*, were obtained from the culture collection maintained at the Microbiology Division at the National Center for Toxicological Research. For each culture, a single colony was grown in Brain–Heart Infusion broth (REMEL Laboratories) overnight at 37°C in an aerobic atmosphere containing 5% CO_2 and the culture was centrifuged at $10\,000 \times g$ for 10 min. The bacterial pellet was re-suspended in sterile milk stabilizer at a concentration of 11.0 $\log_{10}$ CFU/mL.

2.5 Establishment of germfree mouse colonies

GF BALB/c and Tgε26 mice were obtained from the North Carolina State University Gnotobiotic Laboratory. Flexible film isolators (Harlan Isotec, Indianapolis, IN) were erected and sterilized with a solution of 2% peracetic acid and 2.5% sodium dodecylbenzene sulfonic acid (Sigma Chemical, St. Louis, MO). Entry ports were sterilized with peracetic acid solution according to the manufacturer's instructions. Isolator sterility was assessed with weekly swab cultures on trypticase soy agar plates supplemented with 5% sheep blood (REMEL Laboratories). The cultures were incubated at 37°C in a 95% air, 5% CO_2 atmosphere. Standard GF techniques were used to maintain sterile isolator environments. GF mice were introduced into the isolators after establishment of sterile conditions. The mice were fed a sterile NIH-31 diet (PMI Nutrition International, LLC, Brentwood, MO), which contains protein (18%) and complex carbohydrates (5%) that approximates a human diet to facilitate growth of some of the components of the model human microbiota, *ad libitum* [1].

2.6 Colonization of germfree mice

The model human intestinal microbiota containing 8.0–10.0 $\log_{10}$ CFU/mL of each organism was applied with swabs to the oral and anal cavities of the mice and by addition to the food and water in the cages as previously described [25]. When an animal or human is born, a process of microbial succession begins in the gastrointestinal tract that leads to the development of a stable climax community of autochthonous bacteria [18, 19]. The GF mice were colonized with human intestinal bacteria in phases analog to the natural process of microbial succession (Table 1). Microbial succession in humans and mice occurs by a similar mechanism [35]. GF mice were first colonized with species of *Enterococcus*, *Escherichia*, *Bacteroides*, *Pediococcus*, *Lactococcus*, and *Streptococcus.* These bacteria represented the founder community that modifies the intestinal environment to promote colonization by the following groups of bacteria [10, 18]. The second group of bacteria consisted of species of *Lactobacillus*, *Propionibacterium*, *Bifidobacterium*, *Kluyvera*, and *Enterobacteriaceae* [10, 18, 36] that were fed to the mice a day later. On the next day, the third bacterial group was given to the mice and it included species of *Eubacterium*, *Clostridium*, *Fusobacterium*, *Anaerococcus*, *Collinsella*, and *Ruminococcus* [10, 18, 36]. The three mixtures of bacteria that were used for microbial succession of the mice were tested in combination and separately *in vitro* for the ability to provide colonization resistance against *Salmonella* invasion of Caco-2 cells [33].

2.7 Analysis of gastrointestinal (GI) tract colonization

Model human microbiota, probiotic bacteria, and pathogen colonization of the mice were assayed by counting colonies of viable bacteria recovered from feces and from the contents of the small and large intestines. At each phase of microbial succession, fecal samples were collected from three BALB/c mice immediately after elimination and transferred directly to an anaerobic chamber (Coy Laboratories) within 30 min. The samples were homogenized in prereduced brain–heart infusion broth (REMEL Laboratories), serially diluted, and inoculated onto selective agar media [30]. Violet red bile glucose (VRBG) agar for Enterobacteriaceae, mEnterococus agar for enterococci, MRS agar for lactic acid bacteria, and McClueng–Taube (MT) agar for *Clostridium* (REMEL Laboratories). Bifidobacteria were enumerated on *Bifidobacterium* agar (Bifido) and *Bacteroides* spp. were enumerated on *Bacteroides* bile esculin (BBE) agar (Anaerobe Systems). All cultures were grown at 37°C and cultures of Gram-negative facultative anaerobes and eneterococci were grown in an atmosphere of 95% air and 5% CO_2 for 24 h. The obligate anaerobes were cultured anaerobically for 48 h. Small and large intestines, with their contents, were separately homogenized in sterile water at 4°C with a Tissue–Tearor homogenizer (BioSpec Products, Bartlesville, OK) on a setting of no. 2 for 15 s bursts and the homogenates were serially diluted. Fifty μl aliquots were inoculated onto plates of the appro-

priate selective agar medium for the enumeration of various types of bacteria. After the 24 or 48 h incubation, aerobically or anaerobically, at 37°C colonies were counted *per* mL of sample. A 1 mL aliquot of each 5 mL suspension of intestinal contents was dried overnight in a tared aluminum weighing dish at 80°C. The dried dishes were cooled to room temperature and weighed. The final bacterial count was the number of CFU on the plates *per* gram dry weight of homogenate.

2.8 Human enteric pathogen challenge

The natural route of infection in humans by *Salmonella* is by ingestion of contaminated food. To approximate this route of infection, untreated or probiotic-treated HMA mice were orally challenged with either *Salmonella*, or *C. jejuni* at 8.0 $\log_{10}$ CFU/mL on cotton swabs applied to the oral cavities, applied to the surface of feed pellets, and added at 8.0 $\log_{10}$ CFU/mL to the drinking water for 16 h [24]. This method of colonization avoids esophageal trauma, which can cause opportunistic infections in the mice.

2.9 Antigen preparations

Antigens were prepared from lysates of *Salmonella* and *C. jejuni* for *in vitro* activation and apoptosis assays of lymphocytes collected from the spleens of mice from the experiments, as previously described [37]. Briefly, the entire volume of a 500 mL broth culture of bacteria was centrifuged at $2000 \times g$ for 15 min. The bacterial pellet was washed three times with an equal volume of PBS and centrifuged again. The final bacterial pellet was resuspended in 10 mL of PBS and passed through a French pressure cell (Thermo Fisher Scientific, Houston, TX) at 15 000 lb/in^2 to disrupt the bacteria. The disrupted bacteria were centrifuged at $2000 \times g$ and the protein content of the supernatant was determined with a bicinchoninic acid protein assay (Pierce Chemical, Rockford, Ill) and used as the antigens for Western blot analyses and lymphocyte proliferation assays.

2.10 Lymphocyte proliferation assay

Lymphocytes from the spleens of HMA mice with probiotics after *Salmonella* or *C. jejuni* challenge were assayed for proliferative responses to *Salmonella* and *C. jejuni* antigens, as previously described [24]. Lymphocyte proliferation assays were performed with the CellTiter Aqueous 96 assay (Promega, Corporation, Madison, WI). Lymphocytes from the spleens of experimentally treated mice were prepared and incubated at a density of 5×10^5 cells/well of a 96-well culture plate in RPMI medium (Thermo Fisher Scientific, Houston, TX) containing *Salmonella* or *C. jejuni* antigens. Antigens were added to three wells with spleen cells at a concentration of 10 μg whole cell lysate protein antigen preparation *per* well. Antigens were incubated with the cells 56 h at 37°C in a humidified 5% CO_2 incubator before testing for lymphocyte proliferation. The formation of proliferating clonal clusters was also verified microscopically. The proliferation of lymphocytes in response to the antigens was measured as absorbance of reduced 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium (inner salt) at 490 nm, which was measured with a plate reader (Applied Biosystems). The average of three wells *per* sample was used to determine the mean ± SEM Abs_{490} for three mice *per* group. Proliferative responses of lymphocytes to antigens were compared as percent increases in absorbance as a result of the effects of probiotics.

2.11 Apoptosis assay

Lymphocytes from the spleens of the mice 7 days after probiotic treatment and *Salmonella* or *C. jejuni* challenge were analyzed for activation of caspases 3 and 7 (Apo-ONE, Promega Corporation). Each assay well of a Nunc F16 black Maxisorp 96-well fluorescent assay plate (Thermo Fisher Scientific) contained 50 μL of cell suspension in RPMI medium at a cell concentration of 2×10^5 cells/mL to which was added 10 μg antigen preparation *per* well. After 56 h incubation at 37°C in a humidified 5% CO_2 incubator, 100 μL of working substrate solution was added to each well. The plate was rotated at 300 rpm for 30 min at room temperature. Fluorescence intensities were measured in a plate reader (Applied Biosystems) set with filters for an excitation wavelength of 485 nm and an emission wavelength of 530 nm at 30 min intervals until the rates of increase reached a plateau state. Endpoint fluorescence intensities from each treatment group were compared. The percent reduction in relative fluorescence intensities resulting from probiotic inhibition of caspase 3/7 activation were compared.

2.12 Statistical analysis

Three male and three female mice of each mouse strain were used *per* treatment in each experiment, which was the minimum number of samples that could provide 80% statistical power with less than 5% probability of detecting a type 2 error [38]. Evaluation of statistically significant differences between results from treatment groups and control groups were determined with repeated measures one-way analysis of variance and Tukeys post tests using Prism v. 4.0 software (GraphPad Software, San Diego). Nonparametric Kruskall–Wallis and Dunn's tests were conducted on data sets that did not conform to a normal distribution. Numerical count data were $\log_{10}$ transformed prior to statistical analysis to make the data better fit a normal distribution. Statistical significance was defined at $p < 0.05$.

Table 2. Identification of probiotic bacteria

Isolate identification[a)]	Concentration in probiotic product fed to mice (log_{10} CFU/g)
L. acidophilus	6.48
L. rhamnosus	6.48
L. casei	6.48
L. gasseri	6.48
L. reuteri	8.00
B. thermophilus	6.95
B. longum	6.88
B. adolescentis	6.65

a) Probiotic powder was suspended in MRS broth and streaked for isolation on MRS and Bifidobacteria selective media plates. Thirty colonies from each plate were isolated and observed with Gram stains. Six colonies of each morphologically different type from each medium were identified with cellular fatty acid methyl ester composition (Microbial ID) and MicroSeq 16S rRNA sequence homology assays (Applied Biosystems).

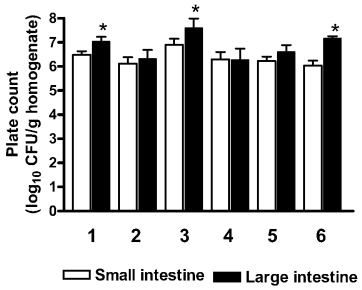

Figure 1. Comparison of microbial groups present in small and large intestines of mice 14 days after colonization succession. Numbers of Gram-negative facultative anaerobes, *Bacteroides* spp., and *Bifidobacterium* spp. were greater in homogenates of the large intestines than small intestines of HMA mice. (1) Gram-negative facultative anaerobes, (2) *Lactobacillus* spp., (3) *Bacteroides* spp., (4) *Enterococcus* spp., (5) *Clostridium* spp., and (6) *Bifidobacterium* spp. Bacteria plate count numbers are mean ± SEM log_{10} CFU/g (dry wt) intestinal homogenates from 6 HMA BALB/c mice. *Bacterial numbers in the large intestinal homogenates were significantly greater than in the small intestines, $p < 0.05$.

3 Results

3.1 Identification of probiotic bacteria

Microbial identification analyses using cellular fatty acid methyl ester composition (Microbial ID) and MicroSeq 16S rRNA sequence homology assays (Applied Biosystems) were used to confirm the presence of bacteria species in the probiotic product. The bacteria were identified as: *L. acidophilus*, *L. rhamnosus*, *L. casei*, *Bifidobacterium thermophilus*, *L. gasseri*, *B. adolescentis*, *B. longum*, and *L. reuteri* (Table 2). The microbes were present in the probiotic product at concentrations ranging from 6.5 to 8.0 log_{10} CFU/g (Table 2).

3.2 Succession colonization of germfree BALB/c mice with the model human microbiota and the effects of adding probiotic bacteria

Dilutions of fecal samples from six BALB/c mice were spread onto selective media plates for colony counts at 1, 2, 3, 7, and 14 days after the first oral inoculation with mixtures (groups) of decreasingly aerotolerant anaerobic bacteria. On the first day after inoculation with bacterial group no. 1, the numbers of enterococci were 6.0 log_{10} CFU/g and the *Bacteroides* spp. and Gram-negative enteric bacteria were 8.0 log_{10} CFU/g feces (Table 3). The day after oral inoculation with bacteria group no. 2, the numbers of enterococci were similar to the numbers of *Bacteroides* spp. at 10.0 log_{10} CFU/g feces. One day after the third bacteria group was orally inoculated the feces from BALB/c mice contained 7.5–8.5 log_{10} CFU/g of all the groups measured. At 7 and 14 days after beginning the colonization succession, all the groups of bacteria were recovered from feces at 7.1–9.4 log_{10} CFU/g. By introducing bacteria into the GI tracts of the mice in a manner similar to natural succession, we were able to achieve a stable presence of representative species from the six groups of bacteria over the 14 day experiment.

Homogenates of the large intestines of BALB/c mice contained significantly more Gram-negative facultative anaerobes, *Bacteroides* spp., and *Bifidobacterium* spp., than the small intestines 14 days after oral inoculation with the model human microbiota (Fig. 1). The cecum sizes of associated mice were not enlarged, as compared with GF mice.

The capacity of the model microbiota and the individual bacterial mixtures used for succession colonization of the mice were tested with an *in vitro* assay for efficacy in colonization resistance against *Salmonella* [33]. Invasion of Caco-2 intestinal cells by *Salmonella* was significantly reduced by each of the succession colonization mixtures, the combined model microbiota, and a commercial competitive exclusion product, which was used as a positive control (Fig. 2).

Seven days after initiating the colonization of BALB/c mice with the model human microbiota, oral inoculation of the mice with 8.0 log_{10} CFU/mL of the probiotic bacteria mixture did not alter the numbers of bacteria groups in the model human microbiota except for significantly more *Bacteroides* spp. in the small intestines of probiotic-treated HMA mice (Fig. 3). Seven days after feeding with probiotic bacteria, the *L. rhamnosus* probiotic was confirmed by culture and microbial identification to be present in cecum homogenates of the gnotobiotic mice as an indicator organism of the persistence of probiotic bacteria in the mice.

Table 3. Colonization succession of GF BALB/c and Tgε26 mice with the model human microbiota

Medium	Numbers of bacteria counted on selective agar media (Mean ± SEM CFU/g (dry wt), $n = 8$) Day 1	Day 2	Day 3	Day 7	Day 14
MRS[a)]	ND[b)]	$4.3 \pm 1.0 \times 10^9$	$1.2 \pm 0.2 \times 11^6$	$1.2 \pm 0.2 \times 10^6$	$3.4 \pm 0.8 \times 10^7$
Bifido	ND[b)]	$2.4 \pm 0.7 \times 10^9$	$1.6 \pm 0.4 \times 10^6$	$1.0 \pm 0.4 \times 10^6$	$3.0 \pm 0.5 \times 10^7$
M-T	ND[b)]	ND[b)]	$3.2 \pm 1.1 \times 10^6$	$3.2 \pm 1.1 \times 10^6$	$1.5 \pm 0.2 \times 10^9$
BBE	$2.1 \pm 1.4 \times 10^8$	$1.8 \pm 1.2 \times 10^{10}$	$2.4 \pm 1.4 \times 10^7$	$1.7 \pm 0.5 \times 10^6$	$7.7 \pm 1.5 \times 10^8$
VRBG	$1.6 \pm 0.9 \times 10^8$	$7.9 \pm 1.5 \times 10^7$	$5.1 \pm 3.0 \times 10^7$	$5.1 \pm 3.0 \times 10^7$	$1.6 \pm 1.0 \times 10^8$
mEnt	$5.2 \pm 1.1 \times 10^6$	$8.9 \pm 2.7 \times 10^9$	$1.7 \pm 0.5 \times 10^6$	$2.4 \pm 1.4 \times 10^7$	$2.8 \pm 0.4 \times 10^8$

a) The mice were orally inoculated with the bacteria mixtures (group 1 on day 0, group 2 on day 1, group 3 on day 2) and feces samples were recovered from four BALB/c and four Tgε26 mice 24 h after each association for quantitative plating on agar plates selective for: *Lactobacillus* spp. (MRS), *Bifidobacterium* spp. (Bifido), *Clostridium* spp. (M–T), *Bacteroides* spp. (BBE), Gram-negative facultative anaerobes (VRBG), and *Enterococcus* spp. (mEnt).

b) ND = Not done, because the mice were not yet associated with these groups of bacteria.

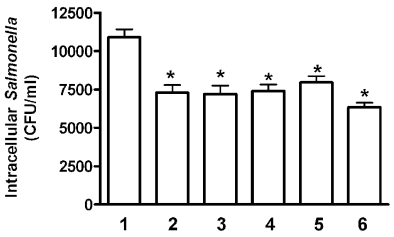

Figure 2. *In vitro* analysis of colonization resistance against *S. enterica* by the model human microbiota. An *in vitro* assay was used to compare the numbers of *S. enterica* cells that invaded Caco-2 cells in 1 h during incubation with (1) media control, (2) a commercial competitive exclusion product, (3) colonization succession bacterial mixture 1, (4) colonization succession bacterial mixture 2, (5) colonization succession bacterial mixture 3, or (6) the combined colonization succession bacterial mixtures. The results are the mean ± SEM CFU/mL intracellular *Salmonella* cells counted from 2 repetitions of three wells *per* treatment. *Significantly fewer intracellular *Salmonella* bacteria than the media control, $p < 0.05$.

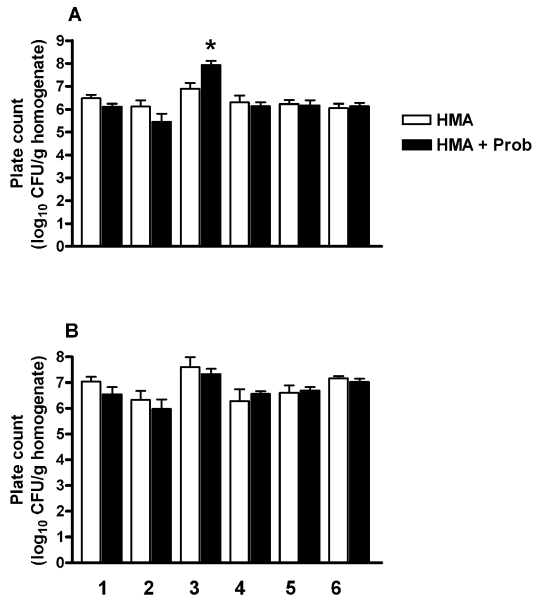

Figure 3. Oral inoculation of HMA mice with probiotic bacteria made little change in the population balance. Homogenates of small (A) and large (B) intestines 7 days after oral inoculation with probiotic bacteria were diluted and plated on selective agar media. Results are from six HMA BALB/c mice and six HMA mice inoculated with probiotic bacteria. (1) Gram-negative facultative anaerobes, (2) *Lactobacillus* spp., (3) *Bacteroides* spp., (4) *Enterococcus* spp., (5) *Clostridium* spp., and (6) *Bifidobacterium* spp. Bacteria plate count numbers are expressed as mean ± SEM $\log_{10}$ CFU/g (dry wt) intestinal contents. Abbreviations: HMA = human microbiota-associated, Prob = probiotic-treated. *Small intestinal bacterial numbers were significantly greater in the HMA + prob mice than in the HMA mice, $p < 0.05$.

3.3 Effects of *Salmonella* and *C. jejuni* challenges

At 1 day after challenge with *Salmonella* or *C. jejuni*, the pathogens were present in feces of BALB/c and Tgε26 mice regardless of the presence of probiotic bacteria (Fig. 4). There were more *Salmonella* and *C. jejuni* in the feces of Tgε26 mice than in the feces of BALB/c mice at 1 day after oral challenge. During the 7 day period of time that the mice were exposed to *C. jejuni* or *Salmonella*, no symptoms of illness were observed.

The probiotic bacteria could not prevent colonization of the small intestines of HMA BALB/c or Tgε26 mice 7 days after challenge with *Salmonella* (Fig. 5A). However, probiotics successfully excluded *C. jejuni* from the small intestines of both strains of mice. The probiotic bacteria could not prevent colonization of the large intestines of HMA BALB/c or Tgε26 mice 7 days after challenge with *Salmonella* (Fig. 5B). However, probiotic bacteria successfully excluded *C. jejuni* from the large intestines of both strains of mice. The probiotic bacteria significantly reduced the number of *S. enterica* in the large intestines of both mouse strains.

3.4 Lymphocyte proliferation and caspase 3/7 activation by *S. enterica* antigens

Proliferative responses of T and B lymphocytes from HMA BALB/c mice to *Salmonella* antigens were not changed as a result of probiotic treatment or after *Salmonella* challenge (Fig. 6). The combination of probiotic treatment and *Salmo-*

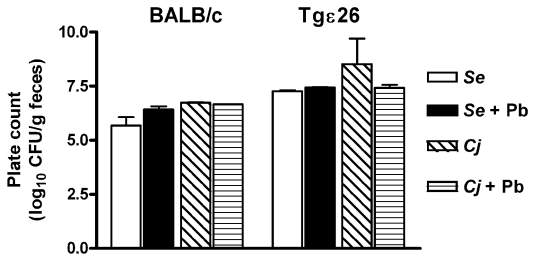

Figure 4. *S. enterica* and *C. jejuni* present in feces 1 day after challenge. *S. enterica* and *C. jejuni* were recovered from the feces of three HMA BALB/c and three Tgε26 mice, 1 day after challenge. Experimental groups were: HMA mice challenged with *S. enterica* (Se) or *C. jejuni* (Cj) and HMA mice treated with probiotics 7 days prior to challenge with *S. enterica* (Se + Pb) or *C. jejuni* (Cj + Pb). Bacteria plate count numbers are expressed as mean ± SEM $\log_{10}$ CFU/g (dry wt) feces.

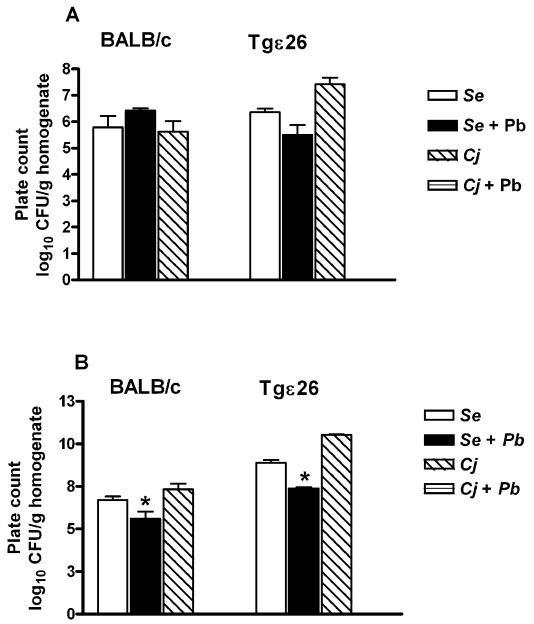

Figure 5. *S. enterica* and *C. jejuni* in the intestines of HMA BALB/c and Tge26 mice 7 days after oral challenge. Homogenates of small intestines (A) from 6 mice of each strain per group were diluted and plated on selective agar media. Experimental groups were: HMA mice challenged with *S. enterica* (Se) or *C. jejuni* (Cj) and HMA mice treated with probiotics 7 days prior to challenge with *S. enterica* (Se + Pb) or *C. jejuni* (Cj + Pb). No *C. jejuni* were detected in the Cj + Pb groups. Bacteria numbers are $\log_{10}$ CFU/g (dry wt) intestinal homogenates. Homogenates of large intestines (B) from six mice of each strain per group were diluted and plated on selective agar media. Experimental groups were: HMA mice challenged with *S. enterica* (Se) or *C. jejuni* (Cj) and HMA mice treated with probiotics 7 days prior to challenge with *S. enterica* (Se + Pb) or *C. jejuni* (Cj + Pb). No *C. jejuni* were detected in the Cj + Pb groups. Bacteria plate count numbers are expressed as mean ± SEM $\log_{10}$ CFU/g (dry wt) intestinal homogenates. *Numbers of *S. enterica* were significantly lower in the Se + Pb group than in the Se group, $p < 0.05$.

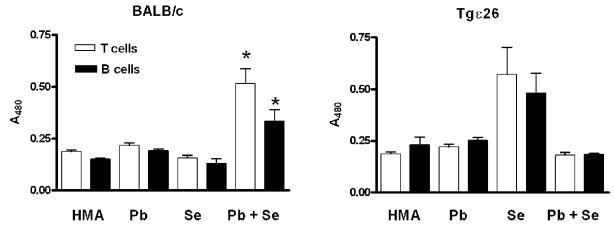

Figure 6. Lymphocyte proliferation to *S. enterica* antigens. The light absorbance at 480 nm was proportional to the number of proliferating lymphocytes in the assay. Comparisons were made of T (open bars) and B (solid bars) cell proliferation from spleens of six HMA BALB/c and six Tgε26 mice (HMA), HMA mice treated with probiotics (Pb), HMA mice orally challenged with *S. enterica* (Se), and HMA mice treated with probiotics and orally challenged with *S. enterica* (Pb + Se). Data are expressed as mean ± SEM absorbance units at a wavelength of 480 nm (A_{480}). *Significantly different from HMA mouse responses, $p < 0.05$.

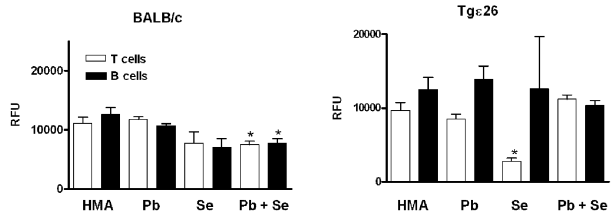

Figure 7. Lymphocyte caspase 3/7 activation by *S. enterica* antigens. Relative fluorescence units (RFU) were proportional to the amount of caspase 3/7 activity in comparisons of T (open bars) and B (solid bars) cells from spleens of six HMA BALB/c and six Tgε26 mice (HMA), HMA mice treated with probiotics (Pb), HMA mice orally challenged with *S. enterica* (Se), and HMA mice treated with probiotics and orally challenged with *S. enterica* (Pb + Se). Data are expressed as mean ± SEM RFU. *Significantly different from HMA mouse responses, $p < 0.05$.

nella challenge of HMA BALB/c mice significantly increased the lymphoproliferative responses of T cells by 68% and B cells by 55% to *Salmonella* antigens. In contrast, the probiotic treatment did not significantly induce Tgε26 spleen cell lymphoproliferation. Some individual Tgε26 mice exhibited enhanced nylon nonadherent (defective T cells) for a 71% increase and adherent (B cells) for a 64% increase in lymphocyte activation after *Salmonella* challenge, but some did not. This variability prevented a possible induction from being statistically significant.

The activation of caspases 3/7 in T and B lymphocytes from HMA BALB/c mice was decreased by 30 and 44%, respectively, 7 days after oral challenge with *Salmonella*, but these reductions were not statistically significant (Fig. 7). A significant reduction of 33 and 38% caspases 3/7 activation occurred in the T and B lymphocytes of probiotic + *Salmonella*-treated HMA BALB/c mice. Caspase 3/7 activation was also decreased 71% in nylon nonadherent T-like lymphocytes from Tgε26 mice. There were marked differ-

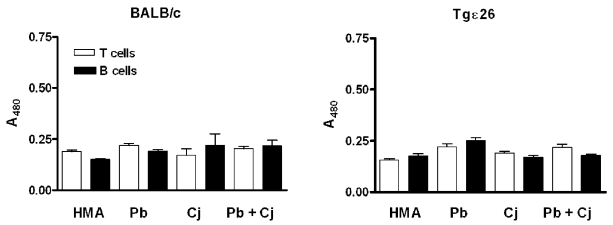

Figure 8. Lymphocyte proliferation to *C. jejuni* antigens. The light absorbance at 480 nm was proportional to the number of proliferating lymphocytes in the assay. Comparisons were made of T (open bars) and B (solid bars) cell proliferation from spleens of six HMA BALB/c and six Tgε26 mice (HMA), HMA mice treated with probiotics (Pb), HMA mice orally challenged with *C. jejuni* (Cj), and HMA mice treated with probiotics and orally challenged with *C. jejuni* (Pb + Cj). Data are expressed as mean ± SEM absorbance units at a wavelength of 480 nm (A_{480}). There were no significant differences between groups, $p < 0.05$.

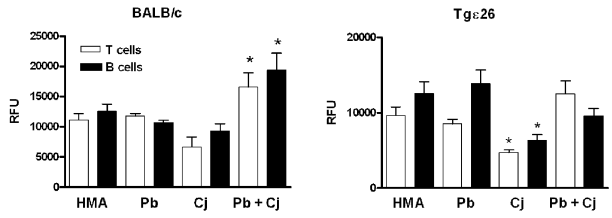

Figure 9. Lymphocyte caspase 3/7 activation by *C. jejuni* antigens. RFU were proportional to the amount of caspase 3/7 activity in comparisons of T (open bars) and B (solid bars) cells from spleens of 6 HMA BALB/c and six Tgε26 mice (HMA), HMA mice treated with probiotics (Pb), HMA mice orally challenged with *C. jejuni* (Cj), and HMA mice treated with probiotics and orally challenged with *C. jejuni* (Pb + Cj). Data are expressed as mean ± SEM RFU. *Significantly different from HMA mouse responses, $p < 0.05$.

ences in caspase 3/7 activation in nylon-adherent lymphocytes of individual *Salmonella*-challenged Tge26 mice.

3.5 Lymphocyte proliferation and caspase 3/7 activation by *C. jejuni* antigens

Treatment and challenge of HMA BALB/c or Tgε26 mice with probiotics, *C. jejuni*, or probiotics + *C. jejuni* appeared to have no significant effect on splenocyte lymphoproliferation by nylon nonadherent or adherent lymphocytes (Fig. 8). The activation of caspases 3/7 in T and B lymphocytes from HMA BALB/c mice treated with probiotics was increased 33 and 35%, respectively, 7 days after oral challenge with *C. jejuni* (Fig. 9). Caspases 3/7 activation decreased 51% in the nylon nonadherent (T-like) and 50% in nylon adherent B lymphocytes from Tgε26 mice 7 days after oral *C. jejuni* challenge. There were marked differences in caspase 3/7 activation in nylon-adherent *versus* nonadherent lymphocytes from HMA Tgε26 mice.

4 Discussion

Probiotic lactobacilli and bifidobacteria competitively exclude *Salmonella* by production of lactic acid and other antibacterial compounds, which can be by direct effects on the pathogens or by immunostimulation of the host [3, 4, 6, 39, 40]. The same capacity to exert a competitive advantage against pathogens may also have an effect on the enteric microbiota. In the present study, the effects of adding probiotics to an established defined microbiota in mice were observed. A change in composition could reduce the capacity for colonization resistance by the microbiota. When the HMA mice in the present study were challenged with 8.0 $\log_{10}$ CFU/mL probiotic bacteria, there was only a significant change in the numbers of *Bacteroides* spp. in the small intestines, but all other bacteria genus groups in the small and large intestines remained stable. Other bacterial groups in the model microbiota were present in relative numbers comparable to a human microbiota [41]. The lack of microbiota population perturbation by probiotics has also been observed in other studies. For example, feeding human subjects 10.5 $\log_{10}$ CFU of bifidobacteria/day did not significantly change the microbiota composition, as detected by denaturing gradient gel electrophoresis [36].

One concern over using an HMA mouse model is that probiotic bacteria intended for use with the human microbiota may not be adapted to survival in the GI tract of a mouse [13]. Using an *in vitro* bioreactor system, Alander *et al.* [30] also observed the presence of lactobacilli and bifidobacteria after addition to a model human microbiota. The addition of probiotics to the model human microbiota disturbed the population balance in their system. In the present study, the probiotics did not cause much change in the microbiota population balance. Probiotic bacteria did not disappear from the feces in mice for the duration of our experiments, but probiotic lactobacilli tend to persist only transiently in the human GI tract [19]. The persistence of allochthonous probiotic bacteria in the present study suggests the reintroduction of organisms to the GI tracts of the mice as a result of the murine tendency towards coprophagy.

In the present study, persistence of the challenge bacteria, *Salmonella* and *C. jejuni*, in HMA mice was evaluated. With oral inocula of 8.0 $\log_{10}$ CFU/mL *Salmonella* and *C. jejuni*, we were able to detect either pathogen in the feces of mice 1 day after challenge of HMA mice. The conventional murine microbiota competitively excludes *C. jejuni*, whereas mice with a limited defined microbiota are readily colonized by this organism [42]. *C. jejuni* also persisted in our model HMA mice. Probiotic bacteria prevented persistence of the HMA mice by *C. jejuni*, but not *Salmonella.* Similar outcomes have been reported for competitive exclusion products, *i. e.*, poultry probiotics, that *Campylobacter*

spp. colonization resistance is more successful than colonization resistance to *Salmonella* [43].

The host has a constantly vigilant mucosal defense system against enteric pathogens that also interacts with the microbiota and any probiotic bacteria that are consumed. The host defenses work together with the microbiota to inhibit growth of GI tract pathogens [35]. By comparing the numbers of pathogenic bacteria in immune-competent and immune-deficient mice, we observed the importance of having an intact immune system and microbiota for protection. There were almost 1 $\log_{10}$ CFU/g more *Salmonella* and *C. jejuni* in the feces of HMA Tgε26 as the HMA BALB/c mice after challenge, which suggests that immunodeficiency can predispose the host to infection by enteric pathogens. We also saw 1–2 $\log_{10}$ CFU/g more *Salmonella* and 2–3 $\log_{10}$ CFU/g more *C. jejuni* in small and large intestines of Tgε26 mice than in BALB/c mice. These results suggest that intact acquired immunity is important for host defense against enteric pathogens; however, small statistically significant changes in bacterial numbers are not as convincing as elimination of the *Salmonella* would have been. However, in the case of *C. jejuni* infection of HMA mice, probiotics and innate defenses were adequate to protect Tgε26 mice from infection.

Colonization resistance against enteric pathogens is one of the major properties probiotics are believed to provide. In the present study, the probiotic bacteria could not prevent the mice from being infected by *Salmonella.* In another study, BALB/c mice fed heat-killed *L. acidophilus* strains had reduced translocation of *Salmonella* to internal organs after oral challenge, but they did not prevent infection of the mice, either [44]. In the same study, *in vitro* experiments showed some possible probiotic effects. The heat-killed mixture of *L. acidophilus* strains was unable to affect the invasion of Caco-2 cells by *S. enterica* serotype Typhimurium but mouse RAW 264.7 cells expressed tumor necrosis factor-α (TNF-α) and had increased nitric oxide production and phagocytic activity in response to the heat-killed lactobacilli. In a different study, culture supernatants from *L. casei*, *L. curvatus*, and *L. plantarum* inhibited *S. enterica* serotype Enteritidis from invading Caco-2 cells [45]. This was concomitant with reduced IL-8 expression by Caco-2 cells. It is apparent that probiotic bacteria can influence the host response to *Salmonella* infection.

The failure of probiotic bacteria to prevent *Salmonella* colonization in the HMA mice during the present study may be due to an immunosuppressive effect of *Salmonella.* Supportive evidence for an immunosuppressive effect of *Salmonella* appeared in our study when lymphocyte proliferative responses to *Salmonella* antigens were not elevated by *Salmonella* infection in BALB/c mice. Probiotic bacteria reversed this apparent immunosuppressive effect of *Salmonella.* Elhofy and Bost [46] have shown that T cell functions are inhibited in immunocompetent mice challenged with *Salmonella.* They reported that macrophage expression of IL-18, which is important for IFN-γ-activated lymphoproliferative responses to intracellular pathogens, was rapidly inhibited after initial stages of infection. An immunosuppressive factor may be produced by *Salmonella* that is involved in its pathogenesis. Sip B for instance, is a recently described candidate [28, 47]. In the presently described experiments, treatment with probiotics reversed the inhibition of lymphocyte proliferation in immunocompetent BALB/c mice. The T cell-deficient Tgε26 mice could not acquire this benefit, which suggests that mature T cells are involved in the immunosuppressive effect of *Salmonella* infection and are involved in the mechanism for probiotic enhancement of lymphocyte antigen responses.

Unlike the results seen with probiotic bacteria + *Salmonella* challenge in BALB/c mice, there was a lack of lymphocyte proliferative effects by probiotics + *C. jejuni*, which can be explained by an effective competitive exclusion of *C. jejuni* by the model human microbiota and the probiotics by 7 days after *C. jejuni* challenge. Since *C. jejuni* was prevented from persistently infecting the HMA BALB/c and Tgε26 mice, no clearly detectable immune response to *C. jejuni* antigens appears to have been mounted. Incidentally, since Tgε26 mice were not infected by *C. jejuni*, the apparent protective effect of the microbiota to this pathogen appeared to not require host immunity. Further studies with adoptive transfer of competent T cells would be needed to completely rule out a role for host immunity in resistance to *C. jejuni*.

One means of control during immune responses is the automatic entry of activated lymphocytes into a state of apoptosis in the absence of specific inhibitory signals. The inhibitory signals activate mechanisms for rescue of the T cells from apoptosis and the persistence of activated T cells prolongs an immune response [48]. Our observation of reduced caspase 3/7 activation in *Salmonella*-challenged HMA BALB/c mice and significantly reduced caspase 3/7 activation in probiotic + *Salmonella*-challenged HMA BALB/c mice suggests that lymphocyte rescue from apoptosis was occurring in our experiments. These results also show that probiotics enhanced this response. The lack of mature T cell control in Tgε26 mice makes it difficult to conclude anything about the caspase activation results from these mice.

While rescue of T cells from apoptosis may be beneficial during a *Salmonella* infection, this probiotic effect may not always be desirable. There is active research into the use of probiotics to control symptoms of inflammatory bowel diseases by attenuating inflammation [35]. Some probiotic bacteria can inhibit T cell proliferation [49], while we report that some can block an antilymphoproliferative effect on T cells by *Salmonella.* Our observation of suppressed T cell apoptosis suggests that probiotics could potentially exacerbate bowel inflammation by inhibiting normal immunomodulating activities in gut tissues. Such unforeseen deleterious effects prompt caution during evalu-

ation of probiotic products for use in some disease processes, perhaps such as inflammatory bowel diseases or as was seen in acute pancreatitis [12].

In conclusion, we report that probiotic lactobacilli and bifidobacteria can supplement the colonization resistance of a model human microbiota against *C. jejuni* enteric persistence in mice. This probiotic effect does not require functional acquired immunity, because immunodeficient Tgε26 mice were protected. The probiotic bacteria were not able to inhibit *Salmonella* persistence in immune-competent or immune-deficient mice; however, inhibition of immunosuppression by *Salmonella* infection by probiotics could improve ultimate recovery from the infection. Results of this study suggest that probiotic bacteria may enhance colonization resistance of the intestinal microbiota to common enteric pathogens.

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The authors have declared no conflict of interest.

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