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Colonization of Congenitally Immunodeficient Mice with Probiotic Bacteria

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We assessed the capacity of four probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei* GG, and *Bifidobacterium animalis*) to colonize, infect, stimulate immune responses in, and affect the growth and survival of congenitally immunodeficient gnotobiotic beige-athymic (*bg*/*bg-nu*/*nu*) and beige-euthymic (*bg*/*bg-nu*/+) mice. The bacteria colonized and persisted, in pure culture, in the alimentary tracts of both mouse strains for the entire study period (12 weeks). Although all adult and neonatal beige-euthymic mice survived probiotic colonization, some infant mortality occurred in beige-athymic pups born to mothers colonized with pure cultures of *L. reuteri* or *L. casei* GG. The probiotic bacteria manifested different capacities to adhere to epithelial surfaces, disseminate to internal organs, affect the body weight of adult mice and the growth of neonatal mice, and stimulate immune responses. Although the probiotic species were innocuous for adults, these results suggest that caution and further studies to assess the safety of probiotic bacteria for immunodeficient hosts, especially neonates, are required.

Probiotics are microorganisms that are ingested to promote beneficial effects on health. An important benefit of probiotics is their capacity to curtail or prevent infectious diseases. For example, studies in humans have shown that probiotic consumption can diminish diarrhea during rotavirus infections (23) and reduce the recurrence of vaginal candidiasis (7). Animal experiments suggest that oral probiotics can interfere with colonization and infection by *Escherichia coli* (4), *Candida albicans* (6, 21, 25), and *Shigella sonnei* (15). Thus, probiotics have great potential as adjuncts or alternatives to conventional antibiotic therapy for infectious diseases.

A serious consideration related to the use of probiotics is whether they are opportunistic pathogens, especially in immunodeficient hosts. Probiotics appear to be innocuous for immunocompetent hosts (1); however, bacteria closely related to probiotic species have been associated with infections in patients. For example, *Streptococcus* spp. and *Lactobacillus* spp. have been isolated from patients with heart valve replacements who have endocarditis (16). Infections by lactic acid bacteria are usually attributed to endogenous flora and not to strains used in foods and probiotic preparations (14). The possible risk of opportunistic infection supports the need for research on the safety of probiotics in immunodeficient hosts.

In this study, we evaluated the capacity of probiotic bacteria to colonize and infect congenitally immunodeficient germfree (GF) beige-athymic (*bg*/*bg-nu*/*nu*) and beige-euthymic (*bg*/*bg-nu*/+) mice.

MATERIALS AND METHODS

Bacteria. We used bacterial cultures that are readily available for use as human probiotics. *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *Bifidobacterium infantis* were obtained from BioGaia Biologics, Inc., Raleigh, N.C. The *B. infantis* strain has subsequently been determined, by ribosomal DNA typing, to be *B. animalis* (12a). *Lactobacillus casei* GG was obtained from Valio, Ltd., Helsinki, Finland. All bacteria were incubated overnight at 37°C in deMan-Rogosa-Sharpe (MRS) medium (Difco, Detroit, Mich.) or on plates of MRS medium with 1.5% agar in anaerobe jars (GasPak; BBL, Cockeysville, Md.) containing anaerobic generators (AnaeroPack System; Carr-Scarborough Microbiologics, Decatur, Ga.). The probiotics were identified and characterized with the API 50CH biochemical identification system (BioMéreiux Vitek, St. Louis, Mo.), and fatty acid analysis was performed by gas-liquid chromatography (Microbial ID, Inc., Newark, Del.).

Mice. NIH *bg*/*bg-nu*/*nu* and *bg*/*bg-nu*/+ mice were obtained from GF breeding stocks maintained at the University of Wisconsin Gnotobiote Laboratory, Madison (http://www.biostat.wisc.edu/gnotolab/gnotolab.html). GF male *bg*/*bg-nu*/*nu* and female *bg*/*bg-nu*/+ mice were housed in sterile flexible-film isolators and were mated to obtain litters of approximately equal numbers of nude and heterozygous mice. A pure culture of each probiotic species was introduced into an isolator containing GF mice. The mice were inoculated by swabbing their oral cavity and anal area with a culture that contained approximately 10^8 viable bacteria per ml. Additional GF mice and newborn mice were colonized by being exposed to feces, feed, and bedding from monoassociated mice. Colonization of the mice was monitored by enumeration of viable bacteria in the feces of gnotobiotic mice. Dilutions of feces were inoculated onto MRS agar plates and incubated at 37°C in anaerobe jars. All the mice were fed autoclaved food and water ad libitum.

Survival and growth of immunodeficient mice colonized with probiotics. Survival of mice born to probiotic-colonized mothers was assessed at 4 and 8 weeks of age. Adult GF mice were colonized with a pure culture of probiotic bacteria, and their body weights and survival were recorded at 4 and 8 weeks after bacterial colonization. Differences in the survival of GF mice and probiotic-colonized mice were evaluated by Kaplan-Meier survival analysis with log rank probability statistics.

Body weights were measured on a Sartorius balance (Brinkman Instruments, Westbury, N.Y.). The body weights of adult mice and the growth rates of newborn mice between 4, 8, and 12 weeks of age were compared with those of GF control mice.

Gastrointestinal tract colonization. Colonization of the gastrointestinal tracts of GF mice with probiotic bacteria was assayed by counting colonies of viable bacteria (CFU) recovered from the contents of the stomachs, small intestines, cecua, and colons of mice that had been sacrificed. The contents were washed out of the organs with sterile water and serially diluted, and 50-μl aliquots were inoculated onto MRS agar plates. The plates were dried briefly and incubated at 37°C overnight in anaerobic jars. 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 number of viable bacteria is expressed as $\log_{10}$ CFU per gram (dry weight) of contents. The pH of the gastrointestinal tract contents was measured with a pH meter and a glass combination electrode (Fisher Scientific Co., Chicago, Ill.).

The spleen, liver, and kidney were aseptically excised, and a portion of each (half a spleen, half a kidney, and a liver lobe) was combined and homogenized in a glass tissue grinder with 5 ml of sterile distilled water, serially diluted, and

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TABLE 1. Viable probiotic bacteria in the gastrointestinal tracts of gnotobiotic mice[a]

Probiotic species	Mouse genotype	Log_{10} CFU of viable bacteria/g (dry wt) of tissues in:				
		Stomach	Small intestine	Cecum	Colon	Feces
L. acidophilus	*bg/bg-nu/nu*	8.2 ± 0.5	9.0 ± 0.6	9.1 ± 0.2	8.1 ± 0.7	9.5 ± 0.2
	bg/bg-nu/+	8.4 ± 0.6	8.8 ± 0.2	9.3 ± 0.4	8.9 ± 0.2	9.9 ± 0.5
L. reuteri	*bg/bg-nu/nu*	8.8 ± 0.1	8.7 ± 0.1	9.5 ± 0.2	9.1 ± 0.1[b]	9.3 ± 0.1
	bg/bg-nu/+	8.9 ± 0.1	8.9 ± 0.1	9.2 ± 0.2	8.8 ± 0.2	9.6 ± 0.1
L. casei GG	*bg/bg-nu/nu*	9.2 ± 0.1	9.0 ± 0.1	9.9 ± 0.1	9.7 ± 0.1[b]	10.0 ± 0.1
	bg/bg-nu/+	9.5 ± 0.1[c]	9.0 ± 0.1	9.9 ± 0.1[d]	9.7 ± 0.1[d]	10.1 ± 0.1
B. animalis	*bg/bg-nu/nu*	8.9 ± 0.3	9.1 ± 0.1	9.5 ± 0.1	9.5 ± 0.1[b]	10.2 ± 0.1
	bg/bg-nu/+	9.1 ± 0.3	9.3 ± 0.1	9.8 ± 0.1[d]	9.7 ± 0.1[d]	9.9 ± 0.1

[a] Samples were taken at 4 to 12 weeks after colonization (11 to 27 mice/group). Values are means ± SEMs.
[b] Significantly greater than *L. acidophilus* ($P < 0.01$).
[c] Significantly greater than *L. acidophilus*, *L. reuteri*, or *B. animalis* ($P < 0.05$).
[d] Significantly greater than *L. acidophilus* and *L. reuteri* ($P < 0.05$).

cultured on anaerobic MRS agar plates overnight at 37°C to detect and quantify the systemic dissemination of the probiotic bacteria. The number of viable bacteria in the internal organs was recorded as CFU per gram (dry weight) of tissue.

Histological evaluations. The alimentary tracts and internal organs of mice that had been sacrificed were fixed in 10% formaldehyde in phosphate-buffered saline (PBS) (pH 7.4). The fixed tissues were dissected, embedded in paraffin, and sectioned (5-μm sections) onto slides for staining with hematoxylin and eosin and Gram stains. Tissue sections of the alimentary tract and the major internal organs were evaluated by a pathologist for evidence of infection and inflammation. Photomicrographs were produced with a Nikon Optiphot microscope (Nikon Inc., Melville, N.Y.) equipped with a Nikon DX-100M automatic camera and a Sony charge-coupled device camera attached to a Targa frame grabber (Truevision, Inc., Indianapolis, Ind.) with Image Pro Plus imaging software (Media Cybernetics, Silver Spring, Md.).

Immune response to probiotics. Immunoglobulin G (IgG), IgA, and IgM concentrations in serum were determined by commercial radial immunodiffusion assays as specified by the manufacturer (The Binding Site, Inc., San Diego, Calif.). Western immunoblots were used to evaluate serum antibody responses to antigens from each of the probiotic bacteria (29). The antigens were prepared from 48-h anaerobic cultures of each bacterial species grown in MRS broth. The entire volume of a 500-ml culture was centrifuged at 2,000 × *g* for 15 min. The bacterial pellets were 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 (SLM/AMINCO, Urbana, Ill.) at 15,000 lb/in^2 to disrupt the bacteria. The disrupted bacteria were centrifuged at 2,000 × *g*, and the protein content of the supernatant was determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.) and used as the antigen for Western blot analyses and lymphocyte proliferation assays.

Antigen preparations (200 μg) were applied to single-lane 4 to 20% polyacrylamide minigels and electrophoresed at 35 mA until the bromphenol blue tracking dye reached the end of the gels. The gels were electroblotted to nitrocellulose membranes, which were incubated in TBS-Tween buffer (0.01 M Tris, 0.15 M NaCl, 0.2% Tween 20 [polyoxyethylene sorbitan monolaurate; Sigma Chemical Co., St. Louis, Mo.])–5% powdered milk for 30 min to block nonspecific antibody binding sites. Pooled serum samples from probiotic-colonized mice were diluted 1:20 in TBS-Tween buffer–1% powdered milk and incubated in lanes on the blots maintained with a Miniblotter-16 (Immunetics, Cambridge, Mass.) for 2 h. The blots were washed with TBS-Tween buffer and incubated for 1 h with alkaline phosphatase-conjugated goat antiserum to mouse IgG, IgA, or IgM (Zymed) diluted 1:1,000. The nitrocellulose strips were washed and incubated with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT/BCIP) substrate solution (Sigma) until bands appeared.

Lymphocyte proliferation assays were performed with the CellTiter Aqueous 96 assay kit (Promega Corp., Madison, Wis.). Splenic lymphocytes were prepared as previously described (29). The spleen cells were pipetted into 96-well culture plates in 200 ml of RPMI 1640 medium (Sigma) with 5% fetal calf serum (BioWhittaker, Walkersville, Md.) at a concentration of 5×10^5 cells/well. Each mitogen or antigen was added to three wells of spleen cells at the following optimized concentrations: 10 μg of lipopolysaccharide (Sigma)/well, 0.5 μg of concanavalin A (Sigma)/well, and 2 to 10 μg of antigen preparation from each probiotic species. The plates were incubated in 5% CO_2 at 37°C for 56 h, 20 μl of Promega MTS/PMS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium and phenazine methosulfate] was added per well, and the plates were incubated for an additional 2 h. The absorbance at 490 nm was measured with an enzyme-linked immunosorbent assay plate reader (Dynatech Laboratories, Inc., Chantilly, Va.), and the absorbance of medium control wells was subtracted from values recorded for each mitogen or antigen. The average result from three wells per sample was used to determine the mean absorbance at 490 nm and standard error of the mean (SEM) for three mice per group.

Statistical analyses. Statistical analyses of the data were performed by a biostatistician (Dennis Heisey, Department of Surgery, University of Wisconsin Medical School, Madison) with SAS software (24).

Kaplan-Meier survival curves were generated to assess differences in survival of *bg/bg-nu/nu* and *bg/bg-nu/+* mice colonized with a probiotic bacterium. Differences between the curves were tested for significance with the log rank test. Two-way analysis of variance with the factors of treatment group and sex was used to assess the effects of probiotics on the growth of mice. Repeated-measures analysis of variance was used to test for differences in the numbers of probiotic bacteria in the gastrointestinal tracts and internal organs of *bg/bg-nu/nu* and *bg/bg-nu/+* mice.

RESULTS

Gastrointestinal tract colonization by probiotics. One inoculation was sufficient to establish the probiotic bacteria in the alimentary tracts of both mouse strains. Weekly cultures of feces from gnotobiotic *bg/bg-nu/nu* and *bg/bg-nu/+* mice demonstrated that both strains of mice were colonized with similar numbers of probiotic bacteria (in the range of 10^8 to 10^{10} CFU/g) and that the mice remained colonized with pure cultures of the probiotics throughout the 12-week study (Table 1). Neither mouse strain eliminated any of the four probiotic bacteria from their alimentary tracts. Also, newborn mice, born to probiotic-colonized mothers, had 10^6 to 10^7 CFU/g in their gastrointestinal tracts by 7 days after birth. At 12 days after birth, they were colonized with adult levels of 10^8 to 10^{10} CFU/g.

Quantitative assessments of viable bacteria in the contents of the stomach, small intestine, cecum, and colon from mice that had been sacrificed were carried out to determine if the probiotic species had different capacities to colonize specific portions of the gastrointestinal tract. Table 1 shows that all of the probiotics colonized the gastrointestinal tracts of *bg/bg-nu/nu* and *bg/bg-nu/+* mice with large numbers of viable bacteria. For all four probiotic bacteria, the cecum contained larger numbers of bacteria than the stomach, small intestine, or colon (Table 1). The pHs of the stomach and intestinal contents from GF mice and probiotic-colonized mice were not significantly different. The stomach pH values were 5.1 ± 0.1 and 5.1 ± 0.7 in GF and probiotic-colonized mice, respectively. Intestinal pH values (6.6 ± 0.6 in the small intestines and 6.9 ±

TABLE 2. Dissemination of probiotic bacteria to internal organs of gnotobiotic mice

Probiotic species	Mouse genotype	Dissemination (%)[a]	No. of bacteria in tissues[b]
None (GF)	*bg/bg-nu/nu*	0	NG[c]
	bg/bg-nu/+	0	NG
L. acidophilus	*bg/bg-nu/nu*	52	6.5 ± 0.8
	bg/bg-nu/+	30	4.9 ± 0.7[d]
L. reuteri	*bg/bg-nu/nu*	0	NG
	bg/bg-nu/+	0	NG
L. casei GG	*bg/bg-nu/nu*	27	4.1 ± 0.4
	bg/bg-nu/+	26	4.1 ± 0.3
B. animalis	*bg/bg-nu/nu*	55	4.4 ± 1.2
	bg/bg-nu/+	31	3.3 ± 0.2[d]

[a] Percentage of mice with systemically disseminated probiotic bacteria (5 to 29 mice/group).

[b] Mean $\log_{10}$ CFU viable bacteria ± SEM per gram (dry weight) of tissues. The tissues cultured included spleen, liver, and kidney from mice colonized for 8 to 12 weeks.

[c] NG, no growth.

[d] The number of bacteria in *bg/bg-nu/+* tissues was significantly less than the number of bacteria in *bg/bg-nu/nu* tissues ($P < 0.05$).

0.3 in the ceca) were similer for both GF and probiotic-colonized mice.

Gram stains of direct tissue impression slides were prepared to assess the incidence of probiotic bacterial adhesion to gastric epithelial cells. Adherence of *L. acidophilus* and *B. animalis* to stomach epithelial cells was observed in 86 and 82%, respectively, of gastric impression Gram stains from both *bg/bg-nu/nu* and *bg/bg-nu/+* mice. *L. reuteri* and *L. casei* GG were observed to be adherent to epithelial cells in only 2 and 5%, respectively, of impression smears from both *bg/bg-nu/nu* and *bg/bg-nu/+* mice respectively ($P < 0.05$; 11 to 23 samples/group). None of the four probiotic bacteria were more adherent to *bg/bg-nu/nu* gastric epithelial cells than to *bg/bg-nu/+* cells.

Translocation (systemic dissemination) of probiotics. Probiotics differed in their capacities to disseminate from the gastrointestinal tract to internal organs. No significant translocation by *L. reuteri* was detected (Table 2). Conversely, translocation to internal organs was detected in 50% and 55% of *bg/bg-nu/nu* mice colonized with *L. acidophilus* or *B. animalis*, respectively (Table 2). The incidence of translocation in *bg/bg-nu/+* mice colonized with *L. acidophilus* or *B. animalis* was 30%. Translocation of *L. casei* GG was also observed in 27% of *bg/bg-nu/nu* and 26% of *bg/bg-nu/+* mice. The numbers of probiotic bacteria cultured from the internal organs of the probiotic-colonized mice ranged from 3.3 $\log_{10}$ CFU/g of homogenized tissues from *B. animalis*-colonized *bg/bg-nu/+* mice to 6.5 $\log_{10}$ CFU/g of homogenized tissues from *L. acidophilus*-colonized *bg/bg-nu/nu* mice (Table 2). The number of *L. acidophilus* or *B. animalis* organisms present in internal organs of *bg/bg-nu/nu* mice was significantly greater than in the organs of the *bg/bg-nu/+* mice. No difference was seen in the number of *L. casei* GG organisms in tissues from *bg/bg-nu/nu* and *bg/bg-nu/+* mice.

Histological evaluations of tissues from probiotic-colonized mice. Tissue sections were obtained from at least six mice colonized with each probiotic species for 4 to 12 weeks. The tissue sections were stained with hematoxylin and eosin and with a Gram stain. In agreement with the direct tissue impression data described above, Gram stains of gastric tissue sections from gnotobiotic mice showed that *L. acidophilus* and *B. animalis* (Fig. 1A) were more adherent to the keratinized epithelium than were *L. reuteri* and *L. casei* (Fig. 1B). Probiotic bacteria were not observed on the secretory epithelium of the stomach.

Histological analysis also revealed abscesses in the stomachs and small intestines of some mice colonized with *L. reuteri* (29%), *L. casei* GG (29%), or *B. animalis* (11%). The abscesses were present in mucosal and submucosal regions of the tissues (Fig. 1C). Histological examinations of tissues from 7- and 12-day-old *L. casei* GG- and *B. animalis*-colonized pups (four of each strain) did not show any evidence of pathologic changes in the gastrointestinal tract or internal organs.

Survival of probiotic-colonized mice. No morbidity or mortality was observed in adult *bg/bg-nu/nu* or *bg/bg-nu/+* mice colonized from 4 to 12 weeks with *L. acidophilus*, *L. reuteri*, *L. casei* GG, or *B. animalis* (Table 3).

Survival of newborn mice, born to mothers colonized with a probiotic species, was also recorded. All *bg/bg-nu/+* and *bg/bg-nu/nu* pups of *L. acidophilus*- or *B. animalis*-colonized mice survived through the 12-week study period. The *bg/bg-nu/+* mice born to mothers colonized with *L. casei* GG all survived during the 12-week evaluation period; however, 36% of *bg/bg-nu/nu* pups born to *L. casei* GG-colonized mothers died before 4 weeks of age (Table 3). All *bg/bg-nu/+* pups born to *L. reuteri*-colonized mothers survived, but 21% of the *bg/bg-nu/nu* pups died.

Effects of colonization with probiotics on reproduction. The litter size averaged from 9 to 10 pups and consisted of roughly equal numbers of *bg/bg-nu/nu* and *bg/bg-nu/+* mice and male and female mice. Colonization with probiotic bacteria did not appear to affect reproduction.

Effects of probiotics on adult body weights. Adult GF mice at least 8 weeks of age, when they had reached mature size, were monoassociated with a probiotic bacterium. The mice were removed from the gnotobiotic isolators at 4, 8, and 12 weeks after colonization with *C. albicans* and weighed. The body weights were compared to those of GF control mice of similar ages to establish whether monoassociation with probiotic bacteria affected their average adult body weights. We observed that adult male *bg/bg-nu/nu* and female *bg/bg-nu/+* mice colonized for 4 to 12 weeks with *L. acidophilus* had body weights that were 60 and 76%, respectively, of the weights of their GF counterparts and that male *bg/bg-nu/nu* mice colonized with *B. animalis* were 87% of the size of the corresponding GF animals (Table 4). Female *bg/bg-nu/nu* and male *bg/bg-nu/+* mice colonized with *L. acidophilus* and female *bg/bg-nu/+* mice colonized with *L. reuteri* or *B. animalis* had significantly greater body weights than did the corresponding GF controls (Table 4).

Effects of probiotics on growth of neonatal mice. Male *bg/bg-nu/nu* pups colonized with *B. animalis*, female *bg/bg-nu/nu* pups colonized with *L. acidophilus*, and female *bg/bg-nu/+* pups colonized with *L. reuteri* were significantly smaller than the GF controls at 8 weeks of age (Table 5). In contrast, male *bg/bg-nu/nu* pups colonized with *L. reuteri* had significantly greater body weights than did the isogenic GF controls (Table 5).

Immune responses. Serum IgM and IgG were induced in *bg/bg-nu/nu* mice by *L. casei* GG and *B. animalis* but not by *L. acidophilus* or *L. reuteri* (Table 6). Production of IgM and IgG in *bg/bg-nu/+* mice was induced by *L. acidophilus*, *L. casei* GG, and *B. animalis* but not by *L. reuteri*. None of the probiotic bacteria were able to induce a significant increase in IgA production in *bg/bg-nu/nu* mice, which is consistent with the lack of

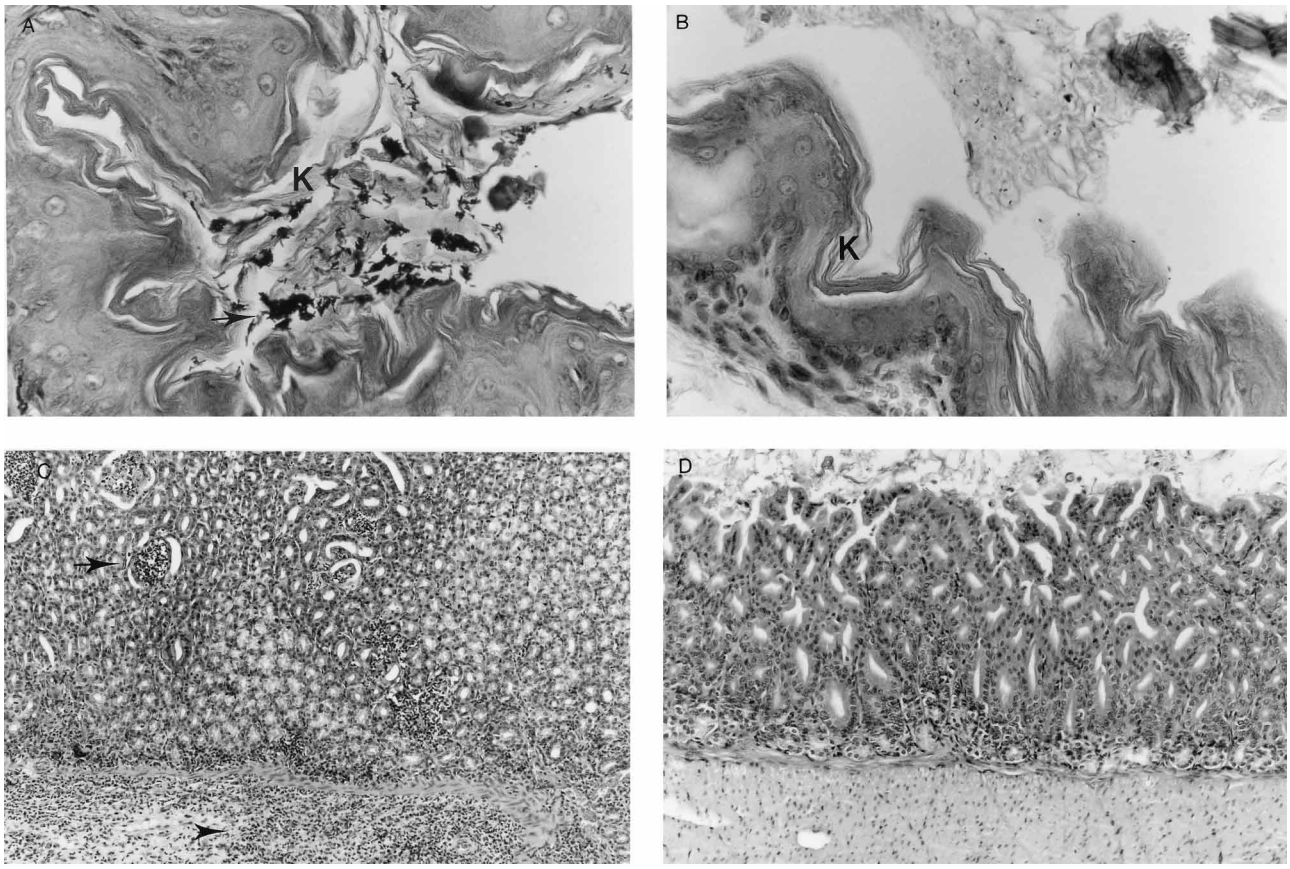

FIG. 1. Histological examination of the gastrointestinal tracts of probiotic-colonized mice. (A) Gram stains of keratinized stomach epithelium from mice colonized with *B. animalis* reveal numerous adherent bacteria (arrowhead) on keratinized, nonsecretory epithelium (K). (B) Fewer adherent bacteria were present in Gram stains of similar preparations from *L. casei*-colonized mice. (C) Mucosal (arrow) and submucosal (arrowhead) abscesses were present in hematoxylin-and-eosin-stained stomach tissues from *B. animalis*-colonized mice. (D) Hematoxylin-and-eosin-stained stomach tissues from GF mice did not contain abscesses. Magnifications, ×240 (A, B, and D) and ×330 (C).

thymus-matured T cells in these mice. IgA production in serum was induced in *bg*/*bg-nu*/+ mice by *L. casei* GG and *B. animalis* (Table 6).

Igs in sera from *bg*/*bg-nu*/+ mice colonized with *L. casei* GG or *B. animalis* not only showed strong specific reactivity with antigens prepared from the bacteria with which the mice were colonized but also had antibodies that cross-reacted with antigens prepared from the other probiotic bacteria (Fig. 2). Conversely, *L. reuteri* colonization did not appear to induce a diversity of either specific or cross-reactive antibodies in the mice (Fig. 2).

Lymphocytes from the spleens of GF and *L. acidophilus*, *L. casei* GG, and *B. animalis*-colonized *bg*/*bg-nu*/+ mice proliferated in response to the B-cell and T-cell mitogens lipopolysaccharide and concanavalin A, respectively, but not to antigens from the probiotic species that colonized the mice (data not shown).

DISCUSSION

The purpose of this study was to assess the pathogenesis of probiotic bacteria for congenitally immunodeficient mice. The probiotic bacteria used in this study were chosen because they were commercially available isolates used in probiotic products. The *L. acidophilus*, *L. reuteri*, and *B. animalis* strains are available as a blend, and the *L. casei* GG isolate has been extensively studied (11–13). The beige nude mice have defects in phagocytic cells and NK cell activity and are without a functional thymus. Our gnotobiotic system showed that the four probiotic bacteria we studied colonized and persisted in very large numbers, in pure culture, in the alimentary tracts of both strains of gnotobiotic mice. In a recent review, McFarland

TABLE 3. Survival of adult and neonatal mice colonized with probiotic bacteria

Mouse and probiotic species	% Survival (total no. of mice per group) for:			
	bg/*bg-nu*/*nu* mice		*bg*/*bg-nu*/+ mice	
	4 wk	8–12 wk	4 wk	8–12 wk
Adult mice				
None (GF)	100 (36)	100 (36)	100 (36)	100 (36)
L. acidophilus	100 (12)	100 (12)	100 (12)	100 (7)
L. reuteri	100 (11)	100 (7)	100 (9)	100 (5)
L. casei GG	100 (12)	100 (5)	100 (12)	100 (12)
B. animalis	100 (10)	100 (7)	100 (14)	100 (14)
Newborn mice				
None (GF)	100 (24)	100 (24)	100 (24)	100 (24)
L. acidophilus	100 (11)	100 (11)	100 (7)	100 (7)
L. reuteri	79 (28)[a]	100 (11)	100 (27)	100 (20)
L. casei GG	64 (53)[a]	100 (23)	100 (56)	100 (39)
B. animalis	100 (14)	100 (11)	100 (22)	100 (16)

[a] Survival was significantly less than that of GF controls by log rank statistics ($P < 0.05$).

TABLE 4. Body weights of adult mice colonized for 4 to 12 weeks with probiotic bacteria

Probiotic species	Body wt (g)[a] of:			
	bg/bg-nu/nu mice		*bg/bg-nu/+* mice	
	Male	Female	Male	Female
None (GF)	32.6 ± 2.3	24.8 ± 0.5	32.7 ± 0.1	28.5 ± 1.0
L. acidophilus	19.6 ± 0.8[b]	26.5 ± 0.5[c]	35.9 ± 0.6[c]	21.8 ± 0.4[b]
L. reuteri	30.3 ± 1.2	21.5 ± 0.8[b]	30.1 ± 3.0	31.8 ± 0.9[c]
L. casei GG	30.6 ± 1.2	24.9 ± 0.6	34.0 ± 0.4	28.0 ± 0.3
B. animalis	28.5 ± 1.6[b]	24.7 ± 0.7	33.2 ± 1.4	33.1 ± 0.3[c]

[a] Mean body weight ± SEM (3 to 14 mice/group).
[b] Significantly less than the GF control ($P < 0.05$).
[c] Significantly greater than the GF control ($P < 0.05$).

and Elmer (13) suggested that a useful biotherapeutic agent must survive transit to an intestinal site, which favors its attachment and multiplication. Our data show that *L. acidophilus*, *L. reuteri*, *L. casei* GG, and *B. animalis* persisted in all sections of the murine alimentary tract, in pure culture, throughout the 12-week study. Persistent colonization by *Lactobacillus* spp. and *Bifidobacterium* spp. in humans has been described previously (8, 11, 20, 22) but usually with the caveat that the bacteria were adapted to a specific host (13). Even though *L. acidophilus*, *L. reuteri*, and *L. casei* GG were of human origin, they apparently had no difficulty in colonizing the alimentary tracts of gnotobiotic mice. The consistent isolation of viable probiotic bacteria from the gnotobiotic mice demonstrates that the four species of probiotic bacteria were capable of colonizing the host's alimentary tract. These latter data suggest that competition with bacteria and/or a varied dietary composition would be major determinants of colonization and persistence of the probiotic bacteria in animals with a conventional microbial flora.

Probiotic bacterial species have occasionally been associated with infectious disease in humans. In one study, *Bifidobacterium* spp. and *Lactobacillus* spp. were isolated from abdominal and reproductive tract abscesses at a frequency of about 4% (15). In another study, 8 of 3,317 blood cultures were positive for *L. casei*, none of which were related to *L. casei* GG (26). Thus, the risk of infection by lactobacilli in the general human population appears to be low; however, little is known of the pathogenesis of lactobacilli in immunodeficient hosts. Our experiments with athymic *bg/bg-nu/nu* mice are among the first to evaluate the pathogenic potential of orally ingested probiotics for congenitally immunodeficient hosts.

That some probiotics can induce pathologic changes in an immunodeficient host was evident in our study because mucosal and submucosal abscesses were detected in the stomachs of some *bg/bg-nu/nu* mice colonized with *L. reuteri*, *L. casei*, or *B. animalis*. Lacking complete NK cell activity, phagocytic cell functions, and T-cell-mediated immunity, *bg/bg-nu/nu* mice may be more dependent on polymorphonuclear leukocytes and macrophages for protection from intestinal microbes. Cell wall components of *L. casei* have been shown to induce inflammatory heart disease in mice, demonstrating that lactobacilli have the potential to induce inflammation (17). Cell wall fragments from *Eubacterium* spp. and *Bifidobacterium* spp. can also induce arthritis in Lewis rats (10). In our study, mice colonized with *L. acidophilus*, *L. reuteri*, *L. casei* GG, or *B. animalis* did not exhibit any ambulatory problems or other signs of arthritis.

Translocation is a term given to the passage of microorganisms from the gut into the internal organs of the host (3). One of the beneficial effects attributed to probiotic bacteria is the inhibition of translocation by pathogenic microorganisms (2, 9). Conversely, the probiotic bacteria may also be able to translocate in immunodeficient hosts. Translocation of *L. acidophilus* and *L. casei* GG in gnotobiotic mice has been previously reported (12). In our experiments, some probiotics (*L. acidophilus* and *B. animalis*) translocated in 50% of the mice examined. The numbers of bacteria that disseminated to the

TABLE 5. Effect of probiotic bacteria on the growth of mice born to probiotic-colonized mothers

Probiotic species	Body wt (g)[a] of:			
	bg/bg-nu/nu mice		*bg/bg-nu/+* mice	
	Male	Female	Male	Female
None (GF)	25.8 ± 1.0	22.6 ± 0.05	30.3 ± 0.9	24.0 ± 0.8
L. acidophilus	25.9 ± 0.4	21.3 ± 0.5[b]	27.8 ± 0.7[b]	22.5 ± 0.7
L. reuteri	29.2 ± 0.2[c]	24.0 ± 0.4	31.0 ± 0.8	22.3 ± 0.5[b]
L. casei GG	26.4 ± 0.2	22.5 ± 0.4	30.7 ± 0.6	25.3 ± 0.8
B. animalis	18.5 ± 3.4[b]	21.5 ± 0.8	28.8 ± 0.2	23.7 ± 0.3

[a] Mean adult body wt ± SEM (3 to 12 mice/group).
[b] Significantly less than the GF control ($P < 0.05$).
[c] Significantly greater than the GF control ($P < 0.05$).

TABLE 6. Igs in the sera of mice colonized with probiotic bacteria

Probiotic species	Mouse genotype	Amt of Ig in mouse sera (μg/ml)[a]		
		IgG	IgA[b]	IgM
None (GF)	*bg/bg-nu/nu*	293 ± 51	<200	28 ± 2
	bg/bg-nu/+	301 ± 123	<200	26 ± 9
L. acidophilus	*bg/bg-nu/nu*	281 ± 38	<200	105 ± 8
	bg/bg-nu/+	729 ± 88[c]	210 ± 10	114 ± 20[c]
L. reuteri	*bg/bg-nu/nu*	68 ± 27	<200	7 ± 4
	bg/bg-nu/+	313 ± 59	<200	38 ± 11
L. casei GG	*bg/bg-nu/nu*	540 ± 120[c]	373 ± 122	414 ± 256[c]
	bg/bg-nu/+	702 ± 96[c]	503 ± 113[c]	84 ± 18[c]
B. animalis	*bg/bg-nu/nu*	2,431 ± 1,651[c]	299 ± 99	399 ± 255[c]
	bg/bg-nu/+	1,792 ± 830[c]	407 ± 56[c]	281 ± 95[c]

[a] Mean ± SEM (five mice/group).
[b] The limit of detection for IgA levels was 200 μg/ml.
[c] Significantly greater than GF control ($P < 0.05$).

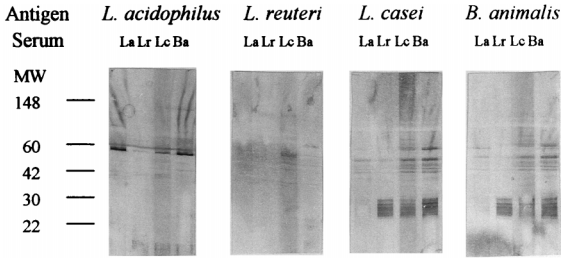

FIG. 2. Western blot of serum antibodies (IgG, IgM, and IgA) from probiotic-colonized mice reacted with probiotic bacterial antigens that were separated by electrophoresis on a 4 to 20% gradient polyacrylamide denaturing gel. Panels contain antigens from *L. acidophilus*, *L. reuteri*, *L. casei* GG, and *B. animalis*. Four lanes across each probiotic protein panel represent immunoblots with pooled antisera (three mice per pool) from mice colonized (4 weeks) with *L. acidophilus* (La), *L. reuteri* (Lr), *L. casei* GG (Lc), or *B. animalis* (Ba). MW, molecular weight in thousands.

internal organs were 6.5 and 3.3 $\log_{10}$ CFU of bacteria per g of homogenized tissues in *bg/bg-nu/nu* and *bg/bg-nu/+* mice, respectively. The incidence of dissemination of *L. acidophilus* and *B. animalis* and the number of viable bacteria per gram of tissue were greater in the athymic *bg/bg-nu/nu* mice than in *bg/bg-nu/+* mice, suggesting that thymus-matured T cells play a role in controlling bacterial translocation. The increased incidence of bacterial adherence to gastric epithelial cells correlated with the incidence of systemic dissemination of the probiotic bacteria. *L. acidophilus* and *B. animalis* adhered to gastric epithelial cells in 86 and 82% of *bg/bg-nu/nu* mice, respectively, and disseminated in 50 and 55% of the mice. *L. reuteri* and *L. casei* GG had 2 and 5% gastric adherence incidence, respectively, and 0 and 27% dissemination, respectively, in *bg/bg-nu/nu* mice. Thus, increased gastric epithelial adhesion correlated with bacterial dissemination to internal organs. Although probiotic bacteria were isolated from internal organs, we did not observe any evidence of increased inflammation or other pathologic findings in tissue sections from mice with systemically disseminated bacteria.

If the probiotics are opportunistic pathogens, they should be able to cause some morbidity, mortality, or pathologic changes in the immunodeficient *bg/bg-nu/nu* mice. No adult *bg/bg-nu/nu* or *bg/bg-nu/+* mice colonized with probiotic bacteria showed outward signs of illness, and none died during the course of these experiments. The probiotic bacteria neither retarded nor enhanced the growth of both male and female (athymic or euthymic) mice. Conversely, there was significant mortality in *bg/bg-nu/nu* pups, by 4 weeks of age, born to mothers colonized with *L. reuteri* or *L. casei* GG; however, no further mortality was evident between 4 and 12 weeks of age. Histological examinations of tissues from 7- and 12-day old *L. casei* GG-colonized pups (four of each strain) did not show any evidence of pathologic changes in the gastrointestinal tract or internal organs. Thus, the reason for the infant mortality in *L. casei* GG-colonized pups remains unknown. We have not come across any other reports of infant mortality by natural or experimental colonization with probiotic bacteria.

Numerous reports suggest that probiotic bacteria can cause immunomodulatory effects that lead to enhanced resistance to enteric pathogens (15, 18, 30). A common immunomodulatory effect of probiotic bacteria is to increase antibody production (18, 30, 31). Probiotic bacteria have been reported to protect mice from infections by gastrointestinal pathogens by increasing IgA production. For example, probiotic-enhanced resistance to *Shigella sonnei* (15), *Salmonella typhimurium*, *Escherichia coli* (19), and rotavirus (30) infections have all been associated with increased IgA production that was apparently induced by feeding probiotic microorganisms.

In our study, all four probiotic bacteria studied induced serum antibodies in euthymic *bg/bg-nu/+* mice, and IgA production was induced by *L. casei* GG and *B. animalis*. Athymic *bg/bg-nu/nu* mice, colonized with a pure culture of the four probiotic species tested, failed to show any significant increases in serum IgA. Serum antibodies were produced by *bg/bg-nu/+* mice colonized with *L. acidophilus*, *L. casei* GG, or *B. animalis* and cross-reacted with antigens from the other probiotic species used in these studies. The cross-reactivity of antibodies to probiotic bacteria suggests the presence of common epitopes on these probiotic bacteria.

There have not been many reports on the effects of probiotic bacteria on cell-mediated immunity. Cell wall extracts from *B. infantis* have been shown to increase experimental delayed-type hypersensitivity responses in mice (27). Interestingly, in the present study we observed no increased proliferation of splenocytes (i.e., greater than the number of spleen cells from GF controls) from mice colonized with *L. acidophilus*, *L. casei* GG, or *B. animalis* upon incubation with antigens from the colonizing bacteria. The induction of cross-reactive antibodies but not lymphoproliferative responses suggests a T-cell-independent activation of immunity to the probiotic bacteria. The lack of a specific in vitro proliferative response suggests that these probiotic bacteria failed to induce specific cell-mediated immunity in *bg/bg-nu/+* mice under these gnotobiotic conditions. In accord with the latter observations, others have also suggested that probiotic species do not induce cell-mediated immune responses in mice (28).

In summary, we have shown that human isolates of *L. acidophilus*, *L. reuteri*, *L. casei* GG, and *B. animalis* can colonize the gastrointestinal tracts of immunodeficient mice. Colonization of *bg/bg-nu/+* mice with *L. acidophilus* and with *L. casei* GG or *B. animalis* in either mouse strain was associated with increased production of antibodies in serum, but we detected no increased lymphocyte proliferation in response to specific probiotic antigens in vitro. Systemic dissemination and induction of mucosal inflammation were indications that the bacteria had infected the mice. Adult *bg/bg-nu/nu* and *bg/bg-nu/+* mice survived colonization, but some infant mortality was associated with colonization by *L. reuteri* or *L. casei* GG. Thus, *L. acidophilus* and *B. animalis* appear to be innocuous probiotics in immunodeficient mice. Overall, probiotic bacteria are likely to be safe for immunocompetent and immunodeficient adults, but they should be tested for safety in immunodeficient neonates.

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