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Biotherapeutic Effects of Probiotic Bacteria on Candidiasis in Immunodeficient Mice

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Four species of probiotic bacteria were assessed for their capacities to protect athymic *bg*/*bg-nu*/*nu* and euthymic *bg*/*bg-nu*/+ mice from mucosal and systemic candidiasis. Each bacterial species and *Candida albicans* colonized the gastrointestinal tracts of both strains of mice. The presence of probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei* GG, or *Bifidobacterium animalis*) in the gastrointestinal tracts prolonged the survival of adult and neonatal *bg*/*bg-nu*/*nu* mice compared to that of isogenic mice colonized with *C. albicans* alone. The incidence of systemic candidiasis in *bg*/*bg-nu*/*nu* mice was significantly reduced by each of the four probiotic bacterial species. The numbers of *C. albicans* present in the alimentary tracts of euthymic *bg*/*bg-nu*/+ mice were significantly reduced by *L. casei* GG and *B. animalis*. None of the probiotic bacteria species completely prevented mucosal candidiasis, but *B. animalis* reduced its incidence and severity. Probiotic bacteria also modulated antibody- and cell-mediated immune responses to *C. albicans*. The prolonged survival of mice, decreased severity of mucosal and systemic candidiasis, modulation of immune responses, decreased number of *C. albicans* in the alimentary tract, and reduced numbers of orogastric infections demonstrated not only that probiotic bacteria have biotherapeutic potential for prophylaxis against and therapy of this fungal disease but also that probiotic bacteria protect mice from candidiasis by a variety of immunologic (thymic and extrathymic) and nonimmunologic mechanisms in this model.

Certain species of lactic acid-producing bacteria are being promoted as probiotics, i.e., live organisms that are ingested to produce beneficial effects on health. Several biotherapeutic effects have been attributed to lactic acid-producing bacteria, including ameliorating lactose intolerance (17, 21), enhancing recovery of a commensal flora after oral antibiotic therapy (28), prophylaxis against and treatment of infant diarrhea (7, 30), and reduction of recurrent urinary tract infections (29).

Candidiasis of oral and vaginal mucosal tissues is very common. For example, nearly 90% of AIDS patients are infected with *Candida albicans* (22). Several studies have assessed the efficacy of probiotics for prophylaxis against and therapy of *C. albicans* infections (2, 6, 12, 32). Vaginitis in apparently healthy women can be caused by *C. albicans*, and the ingestion of yogurt containing *Lactobacillus acidophilus* has been reported to reduce the occurrence of recurrent vaginal candidiasis (12). Laboratory animal studies also suggest that probiotics may be useful for the prevention of candidiasis. Mice immunosuppressed with corticoid drugs recovered more quickly from orogastric candidiasis when they were fed cultures of *L. acidophilus*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* prior to oral *C. albicans* challenge (6). Oral administration of heat-killed *Enterococcus faecalis* prior to oral and systemic infection of cyclophosphamide-treated mice with *C. albicans* prolonged their survival (32).

In this study, we assessed the ability of four probiotic bacterial species, *L. acidophilus*, *Lactobacillus reuteri*, *L. casei* GG, and *Bifidobacterium animalis*, to protect immunodeficient *bg*/*bg-nu*/*nu* and *bg*/*bg-nu*/+ mice from mucosal candidiasis and systemic candidiasis of endogenous (alimentary tract) origin.

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MATERIALS AND METHODS

Microorganisms. Commercial starter cultures of probiotic bacteria *L. acidophilus*, *L. reuteri*, and *Bifidobacterium infantis* were obtained from BioGaia Biologics, Inc., Raleigh, N.C. *B. infantis* has subsequently been determined by ribosomal DNA typing to closely resemble *B. animalis* (20). *L. casei* GG was obtained from Valio, Ltd., Helsinki, Finland. All bacteria were grown overnight 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.) at 37°C. *C. albicans* was cultured on Sabouraud's dextrose agar (SDA; BBL). Microbiological identification and characterization was conducted with the API 50CH biochemical identification system (BioMérieux Vitek, St. Louis, Mo.) and fatty acid analysis by gas-liquid chromatography (Microbial ID, Inc., Newark, Del.).

Mice. C57BL/6 *bg*/*bg-nu*/*nu* mice, which are susceptible to lethal candidiasis (4), and *bg*/*bg-nu*/+ mice, which are resistant to lethal candidiasis (after oral challenge with the pathogenic yeast), were obtained from breeding stocks maintained at the University of Wisconsin Gnotobiote Laboratory, Madison (http://www.biostat.wisc.edu/gnotolab/gnotolab.html). Germfree (GF) male *bg*/*bg-nu*/*nu* and female *bg*/*bg-nu*/+ mice were mated to obtain litters of approximately equal numbers of nude and heterozygous mice. Groups of breeder mice, their progeny, and all adult mice were housed in sterile flexible film isolators and colonized with pure cultures of *C. albicans* or with one of the probiotic species by inoculating their oral and anal orifices with 1 ml (10^7 CFU/ml) of inoculum. Mice colonized with a probiotic species were also inoculated with *C. albicans* (10^7 CFU/ml) for assessment of the effects of probiotics on colonization and infection by *C. albicans*. The microbial colonizations were monitored by quantitative cultures of serially diluted feces collected from mice in the gnotobiotic isolators. Plate dilution cultures of feces were made on anaerobic MRS agar plates that were incubated at 37°C. All mice were given autoclave-sterilized food, water, and bedding ad libitum. Culturing was done weekly to verify the microbial integrity of the experiment.

Survival and growth of immunodeficient mice colonized with probiotics. Survival of mice born to gnotobiotic mothers was assessed at 4 and at 8 to 12 weeks of age. Survival of adult mice was assessed at 4 and at 8 to 12 weeks after colonization with a probiotic bacterium species and *C. albicans*.

Body weights were measured on a Sartorius balance (Brinkman Instruments,

TABLE 1. Probiotic bacteria inhibit *C. albicans* in the gastrointestinal tracts of gnotobiotic mice

Microbial status[e]	CFU of *C. albicans*/g (dry wt) ($\log_{10}$ mean ± SEM)									
	bg/bg-nu/nu mice					*bg/bg-nu/+* mice				
	Stomach	Small intestine	Cecum	Colon	Feces	Stomach	Small intestine	Cecum	Colon	Feces
C. albicans alone	8.0 ± 0.3	8.1 ± 0.4	9.3 ± 0.1	8.6 ± 0.3	9.8 ± 0.1	8.3 ± 0.2	8.0 ± 0.3	9.0 ± 0.2	8.3 ± 0.2	8.6 ± 0.2
C. albicans plus:										
L. acidophilus	7.5 ± 0.3[a]	7.3 ± 0.4[a]	9.0 ± 0.2	7.5 ± 0.3[b]	9.8 ± 0.1	7.9 ± 0.4	7.5 ± 0.4	8.8 ± 0.3	7.8 ± 0.4	8.5 ± 0.1
L. reuteri	9.2[c]	7.9[c]	8.9[c]	7.5[c]	9.5 ± 0.3	7.8 ± 0.2	7.0 ± 0.2[b]	8.4 ± 0.2[a]	7.4 ± 0.2[a]	8.5 ± 0.3
L. casei GG	7.6 ± 0.4	7.9 ± 0.3	8.5 ± 0.3[a]	7.3 ± 0.3[a]	8.4 ± 0.1[a]	6.7 ± 0.1[d]	6.6 ± 0.1[d]	7.0 ± 0.1[d]	6.8 ± 0.2[d]	7.9 ± 0.1[a]
B. animalis	6.4 ± 0.7[b]	7.8 ± 0.3	8.4 ± 0.2[b]	7.3 ± 0.2[d]	8.3 ± 0.1[a]	6.7 ± 0.1[d]	6.6 ± 0.1[d]	7.2 ± 0.3[d]	6.4 ± 0.2[d]	8.3 ± 0.1[a]

[a] Significantly fewer CFU than in *C. albicans*-monoassociated mice (P was <0.05).
[b] Significantly fewer CFU than in *C. albicans*-monoassociated mice (P was <0.01).
[c] Only one mouse was analyzed due to rapid mortality in this group.
[d] Significantly fewer CFU than in *C. albicans*-monoassociated mice (P was <0.001).
[e] For groups of 4 to 21 mice 4 to 8 weeks after colonization.

Westbury, N.Y.). Body weights of adult mice and growth rates of newborn mice between 4 and 8 weeks of age were compared with weights of GF control mice.

Alimentary tract colonization. Probiotic and *C. albicans* colonization of the alimentary tracts of mice was assayed by counting colonies of viable probiotic bacteria (CFU) recovered from feces and from the contents of the stomach, small intestines, cecum, and colon. The contents of the intestines were washed out with sterile distilled water and serially diluted, and 50-μl aliquots were inoculated onto SDA and MRS agar plates. The MRS plates were incubated anaerobically overnight at 37°C. A 1-ml aliquot of undiluted suspension of intestinal contents was dried overnight (80°C) in a tared aluminum weighing dish. The dried dishes were cooled to room temperature and weighed. The numbers of *C. albicans* and probiotic bacteria are reported as $\log_{10}$ CFU/gram (dry weight) of contents. The pH values of alimentary tract washings were measured with a pH meter and a glass combination electrode (Fisher Scientific Co., Chicago, Ill.).

The spleen, liver, and kidneys were aseptically excised, homogenized in glass tissue grinders with 5 ml of sterile distilled water, serially diluted, and cultured on SDA or anaerobic MRS agar plates overnight at 37°C to assess systemic dissemination of *C. albicans* and the probiotics. One milliliter of the tissue homogenate was dried (80°C) overnight to attain the dry weight of the inoculum. The number (CFU) of *C. albicans* in the internal organs is reported as $\log_{10}$ CFU/gram (dry weight) tissue.

Histology. The alimentary tracts and major internal organs of the mice were fixed in 10% formaldehyde in pH 7.4 phosphate-buffered saline (PBS). The fixed tissues were dissected and embedded in paraffin. Five-micrometer sections were placed onto slides and stained with hematoxylin and eosin or a Gram stain. Tissue sections that were taken from representative areas of the alimentary tracts (tongue, palate, esophagus, stomach, small intestine, large intestine, cecum, and colon) and the major internal organs were evaluated by a pathologist for evidence of infection by using the following criteria. Histopathology in infected tissues was scored as follows: 1, 1 to 10 microorganisms (yeast cells and hyphae of *C. albicans*)/high power field at a magnification of ×400 (HPF); 2, 10 to 50 microorganisms/HPF; 3, 50 to 100 microorganisms/HPF; 4, confluent microorganisms/HPF; and 5, confluent microorganisms/HPF with hyphal penetration of viable tissues (yeast cells and hyphae of *C. albicans*). Inflammation, the accumulation of polymorphonuclear and mononuclear cells at mucosal sites of *C. albicans* invasion, was assessed by microscopic examination of hematoxylin-and-eosin-stained sections of infected gastric tissues from four to six *bg/bg-nu/nu* mice that were either monoassociated with *C. albicans* or diassociated with *C. albicans* and a probiotic bacterium species. Photomicrographs were produced with an Optiphot microscope (Nikon Inc., Melville, N.Y.) equipped with a Nikon DX-100M automatic camera and a Sony CCD 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 *C. albicans* and 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, San Diego, Calif.). Western immunblotting was used to evaluate the serum antibody responses to antigens from *C. albicans* (35).

Antigens were prepared from 48-h aerobic cultures of *C. albicans* for Western blot and lymphocyte proliferation assays (5, 35). Antigens were also prepared from 48-h anaerobic cultures of *L. casei* and *B. animalis* for lymphocyte proliferation assays. Briefly, the entire volume of a 500-ml culture was centrifuged at 2,000 × *g* for 15 min. The fungal or bacterial pellets were washed three times with an equal volume of PBS and centrifuged again. The final fungal or 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 fungi or bacteria. The disrupted fungi or 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 antigen for Western blot analyses and lymphocyte proliferation assays.

Antigen preparations (200 μg) from *C. albicans* were applied to a single gel-wide lane of a denaturing 4 to 20% polyacrylamide minigel and electrophoresed at 35 mA until the bromphenol blue tracking dye reached the end of the gel. The separated antigens were electroblotted from the gel onto a nitrocellulose membrane, which was incubated in Tris-buffered saline (TBS)-Tween buffer (0.01 M Tris, 0.15 M NaCl, 0.2% Tween [polyoxyethylene sorbitan monolaurate; Sigma Chemical, St. Louis, Mo.]) and 5% powdered milk for 30 min to block nonspecific antibody binding sites. Pooled serum samples from mice colonized with *C. albicans* and probiotic bacteria were diluted 1:20 in TBS-Tween buffer and 1% powdered milk and incubated in lanes on blots with a miniblotter-16 manfold (Immunetics, Cambridge, Mass.) for 2 h. The blots were washed with TBS-Tween buffer and incubated for 1 h with alkaline phosphatose-conjugated goat antiserum to mouse IgG, IgA, and IgM (Zymed) diluted 1:1,000. The nitrocellulose membranes were incubated with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrate solutions (Sigma) until bands appeared.

Lymphocyte proliferation assays were performed with the CellTiter Aqueous 96 proliferation assay (Promega, Madison, Wis.). Lymphocytes from the spleens of GF, *C. albicans*-monoassociated, or *C. albicans* and probiotic-colonized mice were prepared and incubated at a density of 5×10^5 cells/well in a 96-well culture plate containing mitogens and antigens for 56 h at 37°C in 5% CO_2. Each mitogen or antigen was added to three wells with spleen cells at the following optimal concentrations: 10 μg of lipopolysaccharide (LPS) (Sigma)/well, 0.5 μg of concanavalin A (Sigma)/well, 10 μg of antigen preparation from *C. albicans*, or 2 μg of antigen preparation from *L. casei* or *B. animalis*. The proliferation of lymphocytes in response to mitogens or antigens was proportional to the reduction of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium] after a 2-h incubation at 37°C and 5% CO_2. The reduced MTS was quantified as the spectrophotometic absorbance at a wavelength of 490 nm with a plate reader (Dynatech Laboratories, Inc., Chantilly, Va.). The A_{490} values of three wells per lymphocyte sample from each of three mice were used to calculate the mean ± standard error of the mean (SEM).

Statistical analyses. Statistical analyses of these data were performed by Dennis Heisey, Department of Surgery, University of Wisconsin Medical School, with SAS software (31).

Kaplan-Meier survival curves were generated to assess the significance of observed differences between the survival of *bg/bg-nu/nu* mice colonized with *C. albicans* and that of *bg/bg-nu/nu* mice colonized with *C. albicans* and a probiotic bacterium species. Differences between the curves were tested with the log rank test (33). Repeated measures analysis of variance (ANOVA) was used to test for differences in numbers of viable *C. albicans* in the alimentary tracts or internal organs of mice from the various treatment groups. The data were log transformed to better meet the assumptions of ANOVA. Two-way ANOVA, with factors of treatment group and sex, was employed to detect significant differences in the body weights of probiotic-colonized adult and neonatal mice and to assess significant differences between histopathology severity scores for tissue sections from mice with mucosal candidiasis.

RESULTS

Probiotic suppression of *C. albicans* colonization. Weekly cultures of feces from *bg/bg-nu/nu* and *bg/bg-nu/+* mice housed in gnotobiotic isolators were used to verify that each group of

TABLE 2. Inhibition of systemic candidiasis of endogenous (gastrointestinal tract) origin by probiotic bacteria

Microbial status	*bg/bg-nu/nu* mice		*bg/bg-nu/+* mice	
	Dissemination[a] (%)	No. of *C. albicans*[b]	Dissemination (%)	No. of *C. albicans*
C. albicans alone	75	7.0 ± 0.1	36	6.8 ± 1.2
C. albicans plus:				
L. acidophilus	0[c]	NG[d]	0[c]	NG[d]
L. reuteri	–[e]	–[e]	0[c]	NG[d]
L. casei GG	0[c]	NG[d]	26[c]	4.9 ± 0.8[c]
B. animalis	14[c]	4.6 ± 0.6[c]	12[c]	3.6 ± 0.2[c]

[a] Percentage of mice with disseminated candidiasis (4 to 27 mice/group), euthanized at 4 to 12 weeks after colonization.
[b] Values are expressed as mean ± SEM $\log_{10}$ CFU *C. albicans*/gram of homogenized tissues (spleen, liver, and kidney).
[c] Significantly less than the result for the *C. albicans*-monoassociated control ($P < 0.05$).
[d] NG, no growth.
[e] –, data not available due to early mortality.

mice was continuously colonized with either *C. albicans* alone or with *C. albicans* and one of the probiotic bacteria species. In euthymic *bg/bg-nu/+* mice, *L. casei* GG and *B. animalis* significantly inhibited *C. albicans* throughout the alimentary tract. We recovered as much as 100-fold-fewer CFU of *C. albicans* in diassociated mice than in *C. albicans*-monoassociated mice (Table 1). As shown in Table 1, the number of CFU of *C. albicans* in the stomachs, small intestines, and colons of *bg/bg-nu/nu* mice diassociated with *L. acidophilus* and *C. albicans* was significantly decreased compared with the number in *C. albicans*-monoassociated *bg/bg-nu/nu* mice. The number of viable *C. albicans* was reduced by *L. casei* GG in the ceca, colons, and feces and by *B. animalis* in the stomachs, ceca, colons, and feces of *bg/bg-nu/nu* mice. Neither *C. albicans* nor any of the probiotic bacteria species was eliminated from the alimentary tracts of the mice over the 12-week study. *C. albicans* did not appear to affect the capacity of probiotic bacteria to colonize *bg/bg-nu/nu* or *bg/bg-nu/+* mice, because the numbers of the probiotic bacteria cultured from *C. albicans*- and probiotic bacteria-diassociated mice were very similar to the number cultured from mice monoassociated with a pure culture of the probiotic bacteria (35).

Probiotic inhibition of systemic candidiasis. Compared to *C. albicans* dissemination in mice colonized with only *C. albicans* (75% dissemination in *bg/bg-nu/nu* mice and 36% dissemination in *bg/bg-nu/+* mice), the presence of probiotic bacteria in the alimentary tract reduced the incidence of disseminated candidiasis in both mouse strains (Table 2). Generally, dissemination of *C. albicans* was greater in *bg/bg-nu/nu* mice than in *bg/bg-nu/+* mice; however, with *L. casei* GG the dissemination in euthymic mice was greater than that in athymic mice (Table 2). Overall, these results suggest that thymic and extrathymic immune mechanisms play a role in controlling *C. albicans* dissemination from the alimentary tract (Table 2).

In this study, we also observed that *L. acidophilus*, *L. casei* GG, and *B. animalis* disseminated to internal organs in 4 to 12% of the probiotic- and *C. albicans*-colonized mice. The presence of *C. albicans* in the intestinal tract reduced the incidence of dissemination from 30 to 55% (previously reported for mice monoassociated with a probiotic bacterium species [35]) to 4 to 12%.

Orogastric candidiasis in mice colonized with *C. albicans* and probiotic bacteria. A significant reduction in the incidence and severity of orogastric candidiasis was observed in *bg/bg-nu/+* mice, but not in *bg/bg-nu/nu* mice, colonized with *C. albicans* and *B. animalis* (Table 3). The severity of candidiasis in orogastric tissues (measured as numbers of *C. albicans* observed in keratinized epithelia of the upper alimentary tract) in *bg/bg-nu/nu* mice diassociated with *C. albicans* and *L. acidophilus*, *L. reuteri*, or *L. casei* GG was not significantly less than that in mice monoassociated with *C. albicans* (Table 3).

Inflammation (increased polymorphonuclear leukocyte, monocyte, and lymphocyte infiltration) was evident in *C. albicans*-infected tissues. The presence of *L. acidophilus* or *B. animalis* appeared to increase the incidence of inflammation at sites of *C. albicans* infection. For example, 75% of *bg/bg-nu/nu* mice diassociated with *C. albicans* and *L. acidophilus* and 100% of the mice colonized with *C. albicans* and *B. animalis* had inflammation of their infected gastric tissues, whereas only 30% of *C. albicans*-monoassociated *bg/bg-nu/nu* mice had an obvious inflammatory response in their infected gastric tissues (Fig. 1).

Probiotic bacteria protect immunodeficient mice from lethal candidiasis. All adult *bg/bg-nu/nu* mice died within 2 to 8 weeks after colonization with a pure culture of *C. albicans* (Table 4). In contrast, all adult *bg/bg-nu/+* mice survived monoassociation with *C. albicans*. The survival of adult *bg/bg-nu/nu* mice was significantly prolonged in mice diassociated with *C. albicans* and *L. acidophilus* or *B. animalis* compared to the survival of *C. albicans*-monoassociated *bg/bg-nu/nu* mice (Table 4).

All *bg/bg-nu/nu* mice born to dams that were monoassociated with *C. albicans* died at less than 4 weeks of age. Survival of *bg/bg-nu/nu* pups born to dams colonized with *C. albicans* and a probiotic bacterium species was significantly prolonged compared to that of *C. albicans*-monoassociated mice (Table 4). More protection from lethality was afforded to pups by *L. acidophilus* or *B. animalis* than by *L. reuteri* or *L. casei* GG (Table 4).

Effects of probiotic bacteria on growth of *C. albicans*-colonized mice. Adult GF *bg/bg-nu/nu* mice colonized 4 to 12 weeks with *C. albicans* had lower body weights than GF control mice (Table 5). Generally, adult *bg/bg-nu/+* mice maintained their body weights (compared to GF mice) when colonized with *C. albicans* alone or with a probiotic bacterium species and

TABLE 3. Incidence and severity of orogastric candidiasis in mice diassociated with *C. albicans* and protiotic bacteria

Microbial status	*bg/bg-nu/nu* mice		*bg/bg-nu/+* mice	
	Mucosal infection[a] (%)	Severity score[b]	Mucosal infection (%)	Severity score
C. albicans alone	100	3	83	3
C. albicans plus:				
L. acidophilus	87[c]	3	79	2
L. reuteri	100	5	94	2
L. casei GG	93	3	81	3
B. animalis	100	3	37[c]	1[c]

[a] Percentage of mice with histopathologically confirmed candidiasis of tongue, esophagus, stomach, or hard palate 4 to 12 weeks after colonization (4 to 26 mice/group).
[b] Mean severity score for mucosal candidiasis. Histopathology score in infected tissues was scored as follows: 1, 1 to 10 microorganisms/HPF (magnification, ×400); 2, 10 to 50 microorganisms/HPF; 3, 50 to 100 microorganisms/HPF (yeast cells and hyphae of *C. albicans*); 4, confluent microorganisms/HPF (yeast cells and hyphae of *C. albicans*); 5, confluent microorganisms/HPF with hyphal penetration of viable tissues (yeast cells and hyphae of *C. albicans*).
[c] Significantly decreased from result for *C. albicans*-monoassociated mice, P was <0.05 by repeated measures ANOVA.

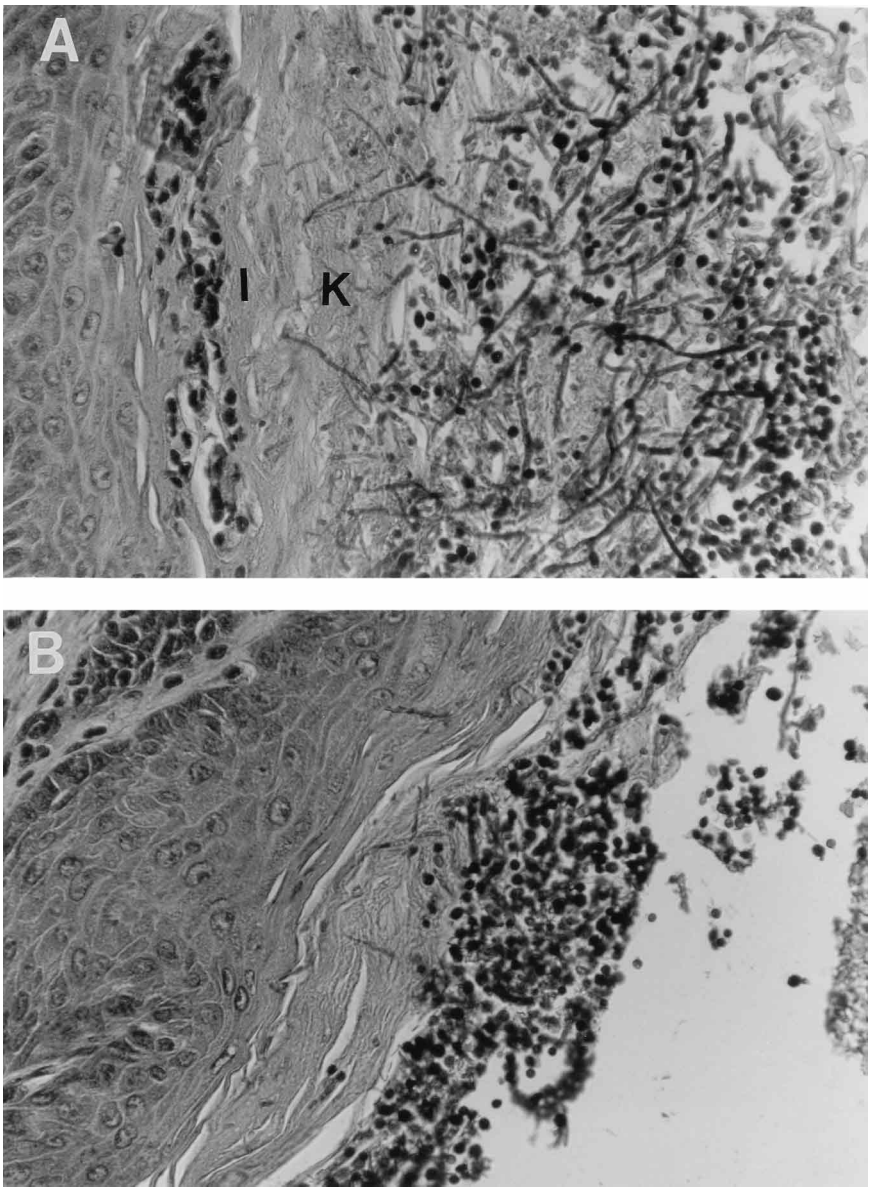

FIG. 1. Induction of inflammation in keratinized stomach tissue by *B. animalis* in *bg/bg-nu/nu* mice with candidiasis. (A) *B. animalis* and *C. albicans* in the outer keratinized layers (K) of the stomach with inflammatory cell infiltrate (I). (B) Lack of an inflammatory infiltrate in *C. albicans*-infected keratinized tissue from *bg/bg-nu/nu* mice colonized with a pure culture of *C. albicans*. Magnification, ×240.

C. albicans; only *bg/bg-nu/+* males diassociated with *C. albicans* and *L. acidophilus* had significantly smaller body weights.

Due to their early deaths, the body weights of *bg/bg-nu/nu* mice born to dams colonized with a pure culture of *C. albicans*, were not compared with the weights of pups born to dams colonized with *C. albicans* and a probiotic. Euthymic *bg/bg-nu/+* mice born to dams monoassociated with *C. albicans* weighed significantly less at 4 and 8 weeks of age than comparable GF mice (Table 6). The euthymic *bg/bg-nu/+* pups born to dams diassociated with *C. albicans* and a probiotic bacterium species weighed significantly less than GF pups at 4 weeks of age, but by 8 weeks their body weights were comparable to those of GF controls.

Modulation of host immune responses to *C. albicans* by probiotic bacteria. Ig isotypes (IgG, IgA, and IgM) were quantified in sera from mice colonized for 4 weeks with *C. albicans* or with *C. albicans* and a probiotic bacterium species (Table 7). Athymic *bg/bg-nu/nu* mice did not produce significant levels of serum IgA except when colonized with *C. albicans* and *B. animalis*; however, serum IgG and IgM were also significantly increased in *bg/bg-nu/nu* mice colonized with *C. albicans* and *B. animalis*. Only IgM was increased in *L. casei* GG- and *C. albicans*-colonized *bg/bg-nu/nu* mice. Interestingly, we observed that the presence of *L. acidophilus* or *L. casei* GG prevented the *C. albicans*-induced increase of serum IgG in *bg/bg-nu/nu* mice. Alimentary tract colonization by *C. albicans* or by probiotic bacteria and *C. albicans* significantly increased IgG, IgA, and IgM in sera from *bg/bg-nu/+* mice over levels in sera from GF mice. (Table 7).

The induction of specific serum Ig (IgG, IgA, or IgM) to *C. albicans* antigens was further investigated by Western blotting analyses. As shown in Fig. 2, sera from *C. albicans*-colo-

TABLE 4. Probiotic bacteria protect immunodeficient mice from lethal candidiasis

Description of mice and microbial status	% Lethality (no. of mice/group) at indicated time of infection			
	bg/bg-nu/nu		*bg/bg-nu/+*	
	4 wk	8–12 wk	4 wk	8–12 wk
Adult				
C. albicans alone	50 (14)	100 (7)	0 (24)	0 (24)
C. albicans plus:				
L. acidophilus	0 (8)[a]	0 (8)[a]	0 (6)	0 (6)
L. reuteri	30 (11)	86 (7)	0 (8)	0 (5)
L. casei	37 (24)	73 (11)	6 (23)	0 (12)
B. animalis	5 (19)[a]	39 (18)[a]	0 (18)	7 (15)
Newborn				
C. albicans alone	100 (15)	–[c]	18 (13)	0 (11)
C. albicans plus:				
L. acidophilus	30 (25)[a]	50 (16)	10 (28)	0 (18)
L. reuteri	70 (35)[b]	100 (10)	8 (26)	0 (6)
L. casei	66 (42)[b]	93 (14)	0 (67)	0 (39)
B. animalis	50 (18)[a]	0 (6)	0 (21)	0 (15)

[a] Significantly decreased lethality compared to that of *C. albicans*-monoassociated control; P was <0.05.
[b] Significantly decreased lethality compared to that of *C. albicans*-monoassociated control; P was <0.01.
[c] –, no data because of early deaths.

nized *bg/bg-nu/+* mice (lane 2) and mice colonized with *C. albicans* and a probiotic bacterium species contained antibodies (IgG, IgA, and IgM) that bound to a variety of *C. albicans* antigens. Also, euthymic *bg/bg-nu/+* mice diassociated with *C. albicans* and *L. casei* GG (Fig. 2, lane 3) had a more diverse serum antibody response to *C. albicans* antigens than did *bg/bg-nu/+* mice diassociated with *C. albicans* and either *B. animalis* (Fig. 2, lane 4), *L. reuteri* (Fig. 2, lane 5), or *L. acidophilus* (Fig. 2, lane 6). A diverse antibody response to *C. albicans* antigens was detected in sera from *bg/bg-nu/nu* mice diassociated with *C. albicans* and either *L. acidophilus* (Fig. 2, lane 9) or *B. animalis* (Fig. 2, lane 10) that was not evident in sera from *C. albicans*-monoassociated *bg/bg-nu/nu* mice (Fig. 2, lane 8) or *bg/bg-nu/nu* mice diassociated with *C. albicans* and *L. casei* GG (Fig. 2, lane 11).

In vitro lymphocyte proliferation assays showed that splenocytes from mice diassociated with *C. albicans* and either *L. casei* GG or *B. animalis* had less of a lymphocyte proliferative (mitogenic) response to LPS than *C. albicans*-monoassociated mice (Table 8). Conversely, lymphocyte prolferation to *C. albicans* antigens was greater with splenocytes from *bg/bg-nu/+* mice diassociated with *C. albicans* and either *L. casei* GG or *B. animalis* than with lymphocytes from *C. albicans*-monoassociated mice (Table 8).

DISCUSSION

All four of the probiotic bacteria species we tested not only prolonged the survival of *bg/bg-nu/nu* mice after oral colonization with *C. albicans* compared with that of *C. albicans* (pure culture)-colonized mice but also decreased the incidence of disseminated candidiasis in both strains (*bg/bg-nu/nu* and *bg/bg-nu/+*) of mice. The presence of a functional thymus was not necessary for the probiotic bacteria to enhance survival and decrease the dissemination of candidiasis in these mice, since the latter protective effect was evident in athymic (*bg/bg-nu/nu*) and euthymic (*bg/bg-nu/+*) mice. Consistent with the latter protective effect, inhibition of systemic dissemination of gastrointestinal pathogens has been described as an attribute of probiotic microorganisms (2, 16).

Few studies have addressed the ability of probiotics to protect immunodeficient hosts from candidiasis. In a previous study, researchers reported that feeding heat-killed *E. faecalis* to mice with cyclophosphamide-induced leukopenia enhanced the recovery of their humoral immune responses to *C. albicans* antigens (32). In another study, *L. acidophilus* and *Streptococcus thermophilus* protected corticosteroid-immunosuppressed mice from systemic (intraperitoneal challenge) candidiasis (6). The latter study involved the use of immunocompetent mice that were treated with immunosuppresive agents to enhance their susceptibility to candidiasis and did not address the efficacy of probiotic protection from candidiasis of endogenous (alimentary tract) origin in congenitally immunodeficient mice.

TABLE 5. Body weights of adult mice colonized for 8 to 12 weeks with *C. albicans* and probiotic bacteria[c]

Microbial status	Mean body weight (g) ± SEM			
	bg/bg-nu/nu		*bg/bg-nu/+*	
	Male	Female	Male	Female
GF	32.6 ± 2.3	24.8 ± 0.5	32.7 ± 0.1	28.5 ± 1.0
C. albicans alone	18.4 ± 2.5[a]	15.2 ± 0.3[a]	31.1 ± 0.6	29.9 ± 3.0
C. albicans plus:				
L. acidophilus	19.0 ± 3.0[a]	18.1 ± 1.0[a,b]	24.1 ± 0.7[a]	29.7 ± 1.1
L. reuteri	–[d]	–[d]	33.2 ± 1.1	31.6 ± 2.5
L. casei GG	14.3 ± 2.6[a]	21.7 ± 4.5	36.4 ± 1.1[b]	29.9 ± 3.0
B. animalis	17.7 ± 0.2[a]	18.6 ± 1.1[a,b]	33.9 ± 1.1[b]	35.6 ± 0.6[b]

[a] Significantly less than the GF control (P was <0.05 by repeated measures ANOVA).
[b] Significantly greater than the result for *C. albicans*-monoassociated mice by repeated measures ANOVA.
[c] Experimental groups contained 3 to 11 mice.
[d] –, data not available due to early mortality and cannibalism.

TABLE 6. Body weights of 4- and 8-week-old *bg/bg-nu/+* mice born to dams colonized with *C. albicans* alone or with probiotic bacteria and *C. albicans*

Microbial status of dam	Body weight (mean ± SEM)[a]			
	Male		Female	
	4 wk	8 wk	4 wk	8 wk
GF	23.8 ± 2.0[c]	30.3 ± 0.9[c]	20.7 ± 1.3[c]	25.8 ± 1.0[c]
C. albicans	7.1 ± 0.6[b]	21.7 ± 2.9[b]	11.7 ± 1.1[b]	19.4 ± 0.5[b]
C. albicans and:				
L. acidophilus	13.0 ± 0.4[b,c]	27.3 ± 0.6	15.1 ± 0.9[b]	22.6 ± 1.2
L. reuteri	18.0 ± 1.6[b,c]	27.7 ± 0.9[c]	15.4 ± 1.9[b]	–[d]
L. casei GG	22.7 ± 1.2[c]	27.6 ± 1.2[c]	16.5 ± 1.9[b]	25.3 ± 1.0[c]
B. animalis	9.6 ± 0.3[b]	24.6 ± 1.5[b]	16.9 ± 2.2	23.8 ± 1.0

[a] Mean body weight (in grams) of *bg/bg-nu/+* mice (3 to 10 mice/group) compared with that of GF mice (24 to 36 mice/group).
[b] Significantly decreased body weights compared to those of GF mice (P was <0.05 by repeated measures ANOVA).
[c] Significantly increased body weights compared to those of *C. albicans*-monoassociated mice (P was <0.05 by repeated measures ANOVA).
[d] –, data not available due to mortality and cannibalism.

TABLE 7. Serum IG (IgG, IgA, and IgM) responses in gnotobiotic mice colonized with probiotic bacteria and/or *C. albicans*

Microbial status	Mean ± SEM (μg/ml) for:					
	bg/bg-nu/nu mice			*bg/bg-nu/+* mice		
	IgG	IgA	IgM	IgG	IgA	IgM
GF	293 ± 51	<200	28 ± 2	301 ± 123	<200	26 ± 9
C. albicans alone	1,936 ± 1,049	229 ± 29	32 ± 7	2,257 ± 121[a]	894 ± 21[a]	54 ± 12
C. albicans plus:						
L. acidophilus	244 ± 25	<200	48 ± 24	1,285 ± 292[a]	761 ± 75[a]	74 ± 5[a]
L. reuteri	–[b]	–[b]	–[b]	1,368 ± 161[a]	437 ± 111	93 ± 59[a]
L. casei GG	233 ± 64	<200	66 ± 8[a]	4,751 ± 1,474[a]	1,526 ± 79[a]	104 ± 36[a]
B. animalis	2,179 ± 367[a]	1,106 ± 39[a]	108 ± 26[a]	3,269 ± 418[a]	1,212 ± 52[a]	155 ± 27[a]

[a] Significantly greater than result for GF control (P was <0.05 by ANOVA). Each group contained five mice.
[b] –, data not available due to early deaths and cannibalism.

Our results show that probiototic bacteria can partially protect congenitally immunodeficient mice from lethal candidiasis.

The four probiotic bacterial species that we studied differed in their biotherapeutic effects on candidiasis. The best overall biotherapeutic effects were observed with *B. animalis*. *B. animalis* prolonged survival compared with that of *C. albicans*-monoassociated controls, decreased systemic dissemination, inhibited *C. albicans* in the alimentary tract, stimulated antibody- and cell-mediated immunity and, in *bg/bg-nu/+* mice, significantly decreased the incidence and severity of orogastric candidiasis. *B. animalis* was more effective as a biotherapeutic agent in mice with a functional thymus than in *bg/bg-nu/nu* (athymic) mice. Our data not only support the importance of a functional thymus in protecting mice against orogastric candidiasis but also demonstrate that *B. animalis* enhanced the resistance of *bg/bg-nu/+* mice to candidiasis to a greater extent than the other three probiotic bacteial species we studied. The role of thymus-matured T cells in resistance to orogastric candidiasis has been well documented (8, 25, 34). Further research is needed to delineate the immune and inhibitory mechanism(s) that enable *B. animalis* to enhance resistance of mice to mucosal and systemic candidiasis.

None of the probiotic strains we tested provided complete protection against candidiasis. It was evident from our studies that suppression of *C. albicans* growth in the intestinal tract by probiotic bacteria was not always associated with enhanced resistance to orogastric candidiasis. We observed that some of the probiotic bacteria inhibited the growth of *C. albicans* in the intestinal tract to some degree; however, the inhibition of *C. albicans* did not always correlate with a reduction in the overall severity of orogastric candidiasis. Two of the probiotics (*L. reuteri* and *L. casei*) are known to produce broad-spectrum antimicrobial compounds, reuterin and caseicin, respectively (1, 24). Volatile fatty acids, such as lactic and propionic acids, and reactive oxygen species, such as H_2O_2, are also produced by probiotics (11, 15), and these molecules may be inhibitory to *C. albicans* (14, 23). Conjugation of bile salts by *Bifidobacterium* spp. is also known to produce antimicrobial substances (9). Thus, production of molecules inhibitory to *C. albicans* may have played a role in decreasing the number of viable *C. albicans* CFU in the alimentary tracts of mice with *L. acidophilus* or *B. animalis*. The fact that *L. acidophilus* and *B. animalis* suppressed the growth of *C. albicans* in vivo better than *L. reuteri* and *L. casei* did suggests that they can produce *Candida*-inhibitory compounds in vivo. Our observation that probiotic inhibition of *C. albicans* growth in the alimentary tract did not always correlate with protection from orogastric candidiasis suggests that probiotic stimulation of host defense (innate and acquired) mechanisms may be more important than bacterial inhibition of *C. albicans* in the intestinal tract in the protection of mice from orogastric or systemic candidiasis.

Our results showed that two strains of probiotic bacteria (*L. acidophilus* and *B. animalis*) enhanced the inflammatory response (consisting of polymorphonuclear leukocytes, macrophages, and lymphocytes) in infected mucosal tissues of *bg/bg-nu/nu* mice. Very little inflammatory cell infiltration was observed in the stomachs of *bg/bg-nu/nu* mice colonized with *C. albicans* (pure culture) or diassociated with *L. casei* GG or *L. reuteri* and *C. albicans*. Thus, *L. acidophilus* and *B. animalis* enhanced the recruitment of inflammatory cells to a *C. albicans*-infected mucosal tissue without the involvement of thymus-matured T cells. The capacity of probiotic bacteria to enhance inflammatory responses likely contributed to the prolonged survival and decreased dissemination of candidiasis we observed in these mice. We are unaware of any other reports on the enhancement of inflammatory cell infiltration by probiotic bacteria in response to an infectious agent.

We observed that either *B. animalis* or *C. albicans* could induce IgA production in *bg/bg-nu/+* mice; however, only

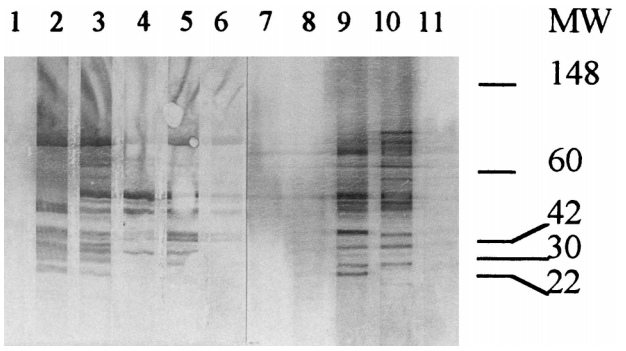

FIG. 2. Antibodies (IgG, IgM, and IgA) to *C. albicans* antigens in mouse sera. Each panel contains a 4 to 20% gradient polyacrylamide denaturing gel electrophoresis of *C. albicans'* antigens. Lanes across *C. albicans'* antigen separations were immunoblotted with pooled antisera (three mice per pool) from GF *bg/bg-nu/+* mice (lane 1); *bg/bg-nu/+* mice colonized with *C. albicans* (lane 2) or diassociated with *C. albicans* and *L. casei* GG (lane 3), *B. animalis* (lane 4), *L. reuteri* (lane 5), or *L. acidophilus* (lane 6); GF *bg/bg-nu/nu* mice (lane 7); *C. albicans*-monoassociated *bg/bg-nu/nu* mice (lane 8); and *bg/bg-nu/nu* mice diassociated with *C. albicans* and *L. acidophilus* (lane 9), *B. animalis* (lane 10), or *L. casei* GG (lane 11). This blot is representative of two experiments with different serum pools. MW, molecular weight (in thousands).

TABLE 8. Proliferation of splenic lymphocytes from *bg/bg-nu/+* mice diassociated with *C. albicans* and probiotic bacteria by antigens from *C. albicans* or probiotic bacteria

Microbial status	Proliferative response (mean A_{490} ± SEM)[a]				
	LPS	Concanavalin A	*C. albicans* antigen	*L. casei* GG antigen	*B. animalis* antigen
GF	0.96 ± 0.01	0.46 ± 0.06	0.02 ± 0.005	0.14 ± 0.04	0.27 ± 0.07
C. albicans alone	0.90 ± 0.03	0.23 ± 0.11	0.27 ± 0.05	0.04 ± 0.02	0.25 ± 0.03
C. albicans plus:					
L. casei GG	0.50 ± 0.02	0.57 ± 0.04	0.48 ± 0.03[b]	0.08 ± 0.005	ND[c]
B. animalis	0.45 ± 0.12	0.46 ± 0.09	0.57 ± 0.21[b]	ND[c]	0.07 ± 0.05

[a] Three to six mice/group.
[b] Significantly greater than result for *C. albicans*-monoassociated mice (P was <0.05).
[c] ND, not done.

C. albicans in combination with *B. animalis* (diassociated) induced IgA production in *bg/bg-nu/nu* mice. IgA production is generally considered to be thymus dependent (26); however, athymic mice are capable of T cell-dependent processes via mucosal T cells of extrathymic origin and maturation (10, 13). Probiotic bacteria are known to enhance antibody responses to pathogens in mice (3). For example, in one previous study, increased antibody production in mice that were fed *Bifidobacterium breve* and infected with rotavirus was reported (36). In other studies, increased resistance and elevated serum antibodies to *Salmonella typhi* were induced by feeding humans *L. acidophilus* (19) and increased resistance and elevated serum antibodies to *Salmonella typhimurium* and *Escherichia coli* were induced by feeding mice *L. casei* (27). Transient increases in IgA (26) and IgG and IgM (18) have also been reported after mice were colonized with *L. acidophilus* or *L. casei*. Our study strongly suggests that *B. animalis*, but not the other three probiotic bacterial species we tested, has the unique capacity to stimulate T cell-dependent IgA and IgG antibody responses in athymic mice, possibly via extrathymic-matured T cells that are present in mucosal tissues.

Our study also showed that in pure culture, *C. albicans* inhibited the growth of *bg/bg-nu/nu* mice. The weight loss appears to be related to the severity of the orogastric infection. *B. animalis* was the most effective probiotic in mice of the four we studied and provided the best overall protection against orogastric and systemic candidiasis; however, we observed that *L. casei* GG and *L. reuteri* were better able than *B. animalis* to counteract the growth-inhibitory effects of *C. albicans* on mice. Thus, *L. casei* GG and *L. reuteri* appeared to produce biotherapeutic effects via nutrient utilization, supplementation, and/or availability. Further study is needed to determine how probiotic bacteria prevent *C. albicans*-induced weight loss.

Overall, this study demonstrated that probiotic bacteria can protect immunodeficient mice from candidiasis; however, none of the probiotic bacteria we studied eliminated *C. albicans* from the alimentary tract or provided complete protection against orogastric and systemic candidiasis. The probiotic bacteria we studied differed in their capacities to prolong survival, inhibit *C. albicans* in the intestinal tract, stimulate antibody- and cell-mediated immunity, and affect the growth rate of gnotobiotic mice. Our data indicate that the probiotic bacteria produced biotherapeutic effects by inhibition of *C. albicans* growth, stimulation of the mucosal and systemic immune systems and possibly by nutritional and competitive means. Of the four probiotic bacterial species that we studied, *B. animalis* was the most biotherapeutic and provided the best overall protection against mucosal and systemic candidiasis. *B. animalis* apparently stimulated host resistance to candidiasis via thymus- and mucosal tissue-associated lymphoid tissues. Overall, thymus and mucosal tissue stimulation by probiotic bacteria strains such as *B. animalis* likely plays a very important role in the enhancement of resistance to infectious agents. More research is needed to elucidate the basic mechanisms utilized by probiotic bacteria so that their beneficial biotherapeutic effects can be optimized.

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