Amelioration of the Effects of \textit{Citrobacter rodentium} Infection in Mice by Pretreatment with Probiotics

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\textbf{Background.} \textit{Citrobacter rodentium} is a naturally occurring murine pathogen that causes colonic epithelial-cell hyperplasia, disrupts the colonic mucosa, and elicits a predominantly T helper 1 cellular immune response; it thereby serves as a model for the study of disease induced by human attaching-effacing pathogens. We sought to determine whether pretreatment of mice with a mixture of \textit{Lactobacillus rhamnosus} and \textit{L. acidophilus} probiotics would attenuate \textit{C. rodentium}-induced colonic disease in mice.

\textbf{Methods.} Mice were administered sterile drinking water, probiotics (10^9 cfu/mL) in sterile drinking water, maltodextrin in sterile drinking water, orogastric \textit{C. rodentium} (10^7 cfu in 0.1 mL), or maltodextrin in sterile drinking water for 1 week before \textit{C. rodentium} infection, or they were pretreated with probiotics (10^9 cfu/mL) for 1 week before challenge with \textit{C. rodentium}.

\textbf{Results.} Mice that received viable probiotics remained healthy. \textit{C. rodentium} infection elicited mucosal inflammation, epithelial-cell hyperplasia, apoptosis in the colon, and interferon (IFN)-\(\gamma\) production by splenocytes. Pretreatment with probiotics decreased levels of all but IFN-\(\gamma\) production.

\textbf{Conclusions.} Pretreatment with probiotics attenuates the effects of \textit{C. rodentium} infection in mice. Understanding the mechanism of these beneficial effects will aid in determining the efficacy of probiotics in preventing infection with related attaching-effacing enteric pathogens in humans.

Probiotics are nonpathogenic, live organisms that interact with host cells to produce beneficial health effects [1, 2]. For instance, lactic acid–producing bacteria are recommended for the treatment of various intestinal disorders, including pouchitis, Crohn disease, traveler’s diarrhea [3, 4], and infectious diarrhea [5, 6].

\textit{Enterohemorrhagic Escherichia coli} (EHEC) O157:H7 causes hemorrhagic colitis and hemolytic-uremic syndrome [7]. Enteropathogenic \textit{E. coli} (EPEC) remains a common cause of diarrhea in infants [8]. Both EHEC and EPEC decrease the transepithelial electrical resistance (TER) of polarized monolayers [9], elicit the secretion of interleukin (IL)–8 [10], and induce reorganization of the cell cytoskeleton [11]. Although these pathogens cause attaching-effacing lesions in rabbits and gnotobiotic piglets, this effect is not readily observed in rodents [12].

\textit{Citrobacter rodentium} is a naturally occurring pathogen of mice that contains a pathogenicity island [13] encoding genes necessary for attaching-effacing lesions [14–16]. \textit{C. rodentium} is the causative agent of transmissible murine colonic hyperplasia [16], crypt cell apoptosis [15], and a predominantly Th1 immune response [17]. \textit{C. rodentium} serves as a small-animal model of attaching-effacing enteropathogens [18] that mimics changes observed in humans with colitis due to Crohn disease [19]. In the present study, we determined whether probiotics ameliorate the severity of colonic disease in mice infected with \textit{C. rodentium}. 

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In vitro analysis of *Citrobacter rodentium* growth in the presence of probiotic culture supernatants or live probiotic bacteria. An inhibition of pathogen-colony formation on MacConkey’s agar plates was seen after 3, 6, or 18 h of coincubation. *C. rodentium* colonization, after coincubation with *Lactobacillus rhamnosus* (A) or *L. acidophilus* (B), was graded as described by Cantey and Hosterman [23], where the intensity of colonization was measured by use of a scale from 0 to 3: 0, no growth; 1, widely spaced colonies; 2, closely spaced colonies; and 3, confluent growth of colonies. The intensity of *C. rodentium* colonization (diamonds) was inhibited, in a time-dependent manner, in the presence of viable *Lactobacillus* species in culture supernatants (squares) or in the presence of culture supernatants alone (triangles). Coculture with viable *Lactobacillus* species in the absence of culture supernatants did not affect *C. rodentium* growth in culture (crosses). Results are the mean ± SE of 3 separate experiments.

**Figure 1.**

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** A commercially available probiotic mixture consisting of *Lactobacillus rhamnosus* strain R0011 and *L. acidophilus* strain R0052 (Lacidofil) was provided by Institut Rosell-Lallemand. Powdered probiotics were rehydrated to a concentration of 10⁷ cfu/L in sterile drinking water. Viability was confirmed by plating 0.1 mL of the rehydrated mixture onto de Man, Rogosa, and Sharpe (MRS) plates supplemented with colistin and nalidixic acid (PML Microbiologicals) that were incubated aerobically for 24 h at 37°C. After inoculation into 10 mL of MRS broth and incubation overnight at 37°C, bacteria were spun at 1600 g for 15 min. Pellets were resuspended to 10⁸ cfu/mL in a 1:1 ratio of Dulbecco’s minimal essential medium and Ham’s F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.6% glutamine, and 1.9% sodium bicarbonate (Gibco BRL).

*C. rodentium* strain DBS 100 (provided by David Schauer, Massachusetts Institute of Technology, Cambridge, MA) [20] was grown under aerophilic conditions on Luria-Bertani (LB) agar plates for 24 h at 37°C, cultured in LB broth for 24 h at 37°C, and spun at 1600 g for 5 min. Pelleted bacteria were resuspended in sterile PBS (Gibco BRL) at 10⁸ cfu/mL.

**Epithelial cell culture.** T84 human colonic epithelial cells [21] were cultured in a 1:1 ratio of Dulbecco’s minimal essential medium and Ham’s F12 supplemented with 10% heat-inactivated FBS (Gibco BRL), 0.6% glutamine, 1.9% sodium bicarbonate, and 2% penicillin-streptomycin (Gibco). Cells were grown in 6.5-mm, 12-well Transwells (Corning) at 37°C in 5% CO₂.

**In vitro growth inhibition assays.** To determine whether probiotics caused growth inhibition, 10⁷ *C. rodentium* in 0.1 mL of sterile PBS was spread onto MacConkey’s agar (PML Microbiologicals), and 10 μL of *L. acidophilus* and *L. rhamnosus* (10⁶ cfu/mL) was spot tested on plates, as described elsewhere [22]. After incubation for 18 h at 37°C, the diameter of the zone of contact inhibition was measured.

In complementary studies, the growth of *C. rodentium* was assessed when it was coincubated with lactobacilli. Lactobacilli (10⁶ cfu/mL) were coincubated at a concentration of 1:1 (vol:vol) with *C. rodentium* (10⁷ cfu/mL) for 3, 6, and 18 h. Aliquots (0.1 mL) were then spread onto MacConkey’s agar plates, and
inhibition was graded on a scale of 0 to 3: 0, no growth; 1, widely spaced colonies; 2, closely spaced colonies; and 3, confluent growth of colonies [23].

Culture supernatants from probiotics were prepared by centrifugation at 1600 g for 5 min and filtering twice through a 0.2-µm filter (Millipore). To determine the effects of culture supernatants on C. rodentium growth, 10^7 cfu of C. rodentium was added to 5 mL of culture supernatants and incubated aerobically for 3, 6, and 18 h at 37°C. The intensity of C. rodentium growth was scored as described elsewhere [23].

**TER.** T84 epithelial cells were grown on 6.5-mm, 12-well Transwells (Corning) at 37°C in 5% CO₂ until TER was >1000 Ohms/cm². Epithelial cells were infected with C. rodentium (10^7 cfu in 2 mL of tissue culture medium) after 3 or 6 h of pre-treatment with L. rhamnosus or L. acidophilus (10^8 cfu in 0.2 mL of tissue culture medium) at 37°C in 5% CO₂. TER was measured after 18 h by use of a Millicell probe (Millipore) [24]. Results are expressed as a percentage of pretreatment TER values.

**Mice.** Forty female C57BL/6 mice, 6 weeks old (Jackson Laboratories), were divided into 6 groups. Mice were provided free access to probiotics in sterile drinking water [25]. Changes in body weight, fluid consumption, and fecal-pellet consistency were used as indications of ill health. Mice were treated as follows. Group A (n = 5) was given sterile drinking water for 19 days; group B (n = 5) was given probiotics (10^9 cfu/mL) in sterile drinking water for 19 days; group C (n = 5) was given maltodextrin in sterile drinking water for 19 days (the vehicle control group); group D (n = 5) was challenged orogastrically with a single dose of C. rodentium (10^7 cfu in 0.1 mL) on day 1 of treatment and given sterile drinking water for 9 days; group E (n = 10) was pretreated with maltodextrin in sterile drinking water for 1 week before challenge with C. rodentium (10^7 cfu in 0.1 mL of sterile PBS) then continued to receive maltodextrin in sterile drinking water for 12 days after infection with C. rodentium; and group F (n = 10) was pretreated with probiotics (10^9 cfu/mL) in sterile drinking water for 1 week before challenge with C. rodentium (10^7 cfu in 0.1 mL of sterile PBS) then continued to receive probiotics in sterile drinking water throughout the duration of the study. Groups E and F were challenged with C. rodentium on 2 separate occasions (n = 5/group).

Fluids were changed on alternate days, and fluid consumption was monitored. Mice were weighed weekly. Colonization by C. rodentium was monitored every 48 h by swabbing the rectum with sterile applicators (Hardwood Products) and plating onto MacConkey’s agar. Mice in groups A, B, and C were killed on day 19 of the study protocol, and those in groups D, E, and F were killed on day 9 after C. rodentium infection. Both groups were killed by an intraperitoneal injection of 0.1 mL of chloral hydrate (Sigma-Aldrich Canada). Animal care and interventions were performed according to a protocol approved by the Laboratory Animal Services at the Hospital for Sick Children.

**Tissue collection.** Sections of the distal colon were excised and transferred into sterile PBS for bacterial colonization, into universal fixative (4% paraformaldehyde/1% gluteraldehyde in a 1:1 ratio [pH 7.0]) for subsequent electron microscopic analysis, or into neutral-buffered formalin for histopathological analysis or were snap frozen in liquid nitrogen for TUNEL analysis.

**Measurement of bacterial colonization of the intestinal tract.** Rectal swabs were plated onto MacConkey’s agar and incubated aerobically for 24 h at 37°C. Bacterial populations were graded according to the method described by Cantey and Hosterman [23]. C. rodentium colonies were easily distinguished by their size and morphologic appearance [26].

**Bacterial populations in the colon.** Luminal contents were removed from a segment of colon and serially diluted in PBS, plated onto MacConkey’s agar, and incubated aerobically for 24 h at 37°C. Bacterial colonization was assessed by colony morphology and intensity of growth; this was graded on a scale of 0 to 3 [23].
Histopathological analyses. Coded sections of colonic tissues were stained with hematoxylin-resin and assessed for bacterial colonization, morphological changes, and the degree of epithelial-cell hyperplasia. Colonic sections were assessed for the severity of mucosal inflammation (score, 0; scant foci; 1, minimal foci; 2, minimal to mild foci; 3, moderate to severe foci; 4, severe apical inflammation) [27] by a single blinded observer (B.Y.N.). Epithelial thickness was measured by micrometry by use of a Leica NEWDM 4500BR microscope: 15 measurements were taken of each sample and are expressed in micrometers.

Apoptosis of colonic epithelia. TUNEL analysis was performed to quantitate the apoptotic colonic epithelium [28] (Discovery; Ventana Medical Systems). Positive stained cells in coded sections were counted, by a single blinded observer (Y.A.), in at least 10 well-oriented sections. Results are expressed as the number of positively stained cells per 10 colonic crypts (± SE).

As a complementary assay, caspase-3 activation was determined by immunohistochemical analysis [29]. Tissue sections were baked overnight at 60°C, dewaxed in xylene, and hydrated in distilled water through decreasing concentrations of alcohol.

Rabbit anti–human CPP32 (Dako) was used on an autoimmunostainer (Ventana Medical Systems) at a dilution of 1:250. Immune detection was done at a 1:100 dilution of biotinylated anti–rabbit IgG (Vector Laboratories) conjugated with Ventana iVIEW 3–3′-diaminobenzidine (Vector). Endogenous biotin was blocked, and sections were counterstained with hematoxylin. Positive staining of coded sections was assessed under brightfield microscopy.

Transmission electron microscopy. Sections of colon were fixed in universal fixative (4% paraformaldehyde:1% gluteraldehyde in a 1:1 ratio [pH 7]). Sections were incubated in osmium tetroxide for 1 h, dehydrated in a graded series of ethanol concentrations (50%–100%), and embedded in epoxy resin [30]. Ultrathin sections (80 nm) were cut on a Reichert Ultracat E machine (Leica) and viewed by use of a JEM-1230 (JOEL USA) microscope.

Preparation of splenocytes for cytokine measurements. Spleens were removed and prepared as described elsewhere [31]. Single-cell suspensions were obtained by mashing spleens through sterile filters in RPMI 1640 culture medium and centrifugation at 800 g for 5 min. Cells were then incubated in 2 mL of erythrocyte lysis buffer for 2 min, and the cell pellet was layered with 5 mL of lympholyte-M (Cedarlane) and washed 3 times in RPMI culture medium. Isolated splenocytes were resuspended (10⁶ cells/mL) and incubated with sterile C. rodentium whole-cell sonicate for 72 h at 37°C. Cytokine production was then measured by use of commercial immunoassay kits, according to the manufacturer’s instructions (Biosource).

Statistical analyses. Results are expressed as the mean ± SE of ≥3 separate experiments. Comparisons were performed by use of 2-way analysis of variance (ANOVA) and the unpaired Student’s t test.

RESULTS

In vitro inhibition of C. rodentium growth by lactobacilli. C. rodentium grown on MacConkey’s agar and exposed to the individual Lactobacillus strains did not produce zones of contact inhibition (data not shown). Incubation of C. rodentium with either L. rhamnosus (figure 1A) or L. acidophilus (figure 1B), in the presence of probiotics plus culture supernatants or that of culture supernatants alone, inhibited C. rodentium growth in a time-dependent manner. In contrast, bactericidal effects were not observed when C. rodentium was coincubated with L. rhamnosus in the absence of probiotic culture supernatants. The maximum activity against C. rodentium was observed when both probiotics and culture supernatants were present in coinoculation or incubation medium. The pH of coculture medium (pH 6.5) did not differ from that of C. rodentium alone (pH 6.5), which suggests that growth inhibitory effects were mediated through a bacterial product secreted into the culture supernatant. Pretreatment with lactobacilli for 3 or 6 h before

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Table 1. Precolonization with probiotics decreases Citrobacter rodentium colonization.

<table>
<thead>
<tr>
<th>Treatment (no. of mice)</th>
<th>C. rodentium cultured from rectal swabs, score 0–3</th>
<th>Colonic luminal C. rodentium load, score 0–3</th>
<th>Mice with colonization of the colon score of 3, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Probiotics (5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maltodextrin (5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. rodentium (5)</td>
<td>3</td>
<td>3⁰</td>
<td>80</td>
</tr>
<tr>
<td>Maltodextrin + C. rodentium (10)</td>
<td>3</td>
<td>3⁰</td>
<td>80</td>
</tr>
<tr>
<td>Probiotics + C. rodentium (10)</td>
<td>3</td>
<td>1.6 ± 0.2⁰</td>
<td>30⁰</td>
</tr>
</tbody>
</table>

NOTE. Rectal swabs plated onto selective MacConkey’s agar plates from all mice infected with C. rodentium had bacterial scores of 3. The load of C. rodentium present in the lumen of the colon was reduced in mice that received probiotic pretreatment. The percentage of mice presenting with severe [23] (score, 3) C. rodentium colonization was reduced from 80% in infected mice to 30% in mice pretreated with probiotics.

⁰ P<.0001, Student’s t test.
Figure 4. Reduction of colonic epithelial-cell hyperplasia induced by *Citrobacter rodentium* infection after pretreatment with probiotics. A and B, Normal morphological characteristics of the colon in control mice and those given probiotics, respectively, without subsequent pathogen challenge. Double-headed arrow, Crypt height, measured by use of a micrometer. C, *Citrobacter rodentium*-induced epithelial hyperplasia, as indicated by the double-headed arrow. Also evident are the ragged edges and increased cellular sloughing observed in the colon after *C. rodentium* infection in mice. D, Decrease in colonic epithelial thickness in C57BL/6 mice pretreated with probiotics before *C. rodentium* infection vs. tissue obtained from *C. rodentium*-infected mice not pretreated with probiotics. (hematoxylin-eosin stain; original magnification, ×60). Quantitative analysis of colonic epithelial hyperplasia shows a reduction in severity of hyperplasia in mice pretreated with probiotics before challenge with *C. rodentium*. E, Crypt height of colonic tissue from control mice, probiotic-treated mice, mice treated with maltodextrin, mice infected with *C. rodentium*, mice pretreated with maltodextrin before challenge with *C. rodentium*, and mice pretreated with probiotics before challenge with *C. rodentium*. Results are mean ± SE. *P < .001, **P > .05 vs. C. rodentium.*
treated mice, vs. untreated control mice (mprobiotics reduced epithelial thickness to in crypt height ( in the lamina propria (, ANOVA). With pretreatment with dentium infection caused an increased cellular infiltrate into pretreatment with probiotics. As shown in figure 5, C. rodentium treatment with probiotics.

C. rodentium in the lamina propria of C57BL/6 mice, compared with mice treated with probiotics alone. Probiotic pretreatment before challenge with C. rodentium resulted in a significant decrease in the lamina propria inflammation toward control levels. Results are mean ± SE. *P < .05 vs. probiotic treated mice, **P < .05 vs. C. rodentium.

infection did not attenuate C. rodentium–induced decreases in the TER of T84 monolayers (figure 2).

Safety of viable probiotics. Over the duration of the study protocol, all mice remained healthy. Fluid consumption in each of the 3 treatment groups did not differ from that in the 3 control groups (figure 3A). Body weights of mice in each of the 6 study groups were not different (figure 3B).

Decreased C. rodentium colonization of the colon after pretreatment with probiotics. C. rodentium was recovered in rectal swabs from all of the infected mice over the entire duration of the study (table 1). The C. rodentium load in luminal contents from a 2.0-cm section of colon after death was lower in probiotic-treated mice than in untreated mice (P < .0001). The percentage of mice with a C. rodentium colonization score of 3 was reduced from 80% to 30% when mice were pretreated with probiotics.

Reduction in colonic hyperplasia in C. rodentium–infected mice after pretreatment with probiotics. There was an increase in epithelial thickness in mice infected with C. rodentium (212.6 ± 8.5 μm) (figure 4C and 4E), compared with that in untreated control mice (99.8 ± 7.2 μm) (figure 4A) (P < .001, ANOVA). Probiotic treatment alone did not cause an increase in crypt height (80.4 ± 5.3 μm) (figure 4B). Pretreatment with probiotics reduced epithelial thickness to 131.0 ± 8.4 μm (figure 4D and 4E) (P < .001, ANOVA).

Decrease in colitis induced by C. rodentium infection after pretreatment with probiotics. As shown in figure 5, C. rodentium infection caused an increased cellular infiltrate into the lamina propria (P < .05, ANOVA). With pretreatment with probiotics, there was a decrease in the cellularity of the lamina propria toward levels in control mice (P < .05, ANOVA). The administration of maltodextrin had no effect on mucosal inflammation in response to C. rodentium infection.

Reduction in epithelial cell damage induced by C. rodentium infection after pretreatment with probiotics. Pretreatment with probiotics was beneficial in maintaining cellular morphological characteristics (figure 6). Electron photomicrographs of colon sections from probiotic-treated mice showed normal cellular morphological characteristics, with intact microvilli and fusiform bacteria present in the gut lumen (figure 6A). Similar images were obtained from untreated control mice. C. rodentium infection disrupted cellular morphological characteristics, with subnuclear membrane vacuoles and disruption of the nuceloplasm, loss of goblet cells, and an increase in bacterial internalization (figure 6B and 6C). Chromatin condensation in the nuclei of numerous epithelial cells, which is indicative of increased apoptosis, was also observed. Pretreatment with probiotics decreased bacterial internalization, re-stored goblet-cell formation, and increased mucus secretion. However, shortened microvilli and reduced changes in nuclear chromatin levels were still evident (figure 6D).

Increased apoptosis of epithelial cells in C. rodentium–infected mice. TUNEL-stained colon sections from control mice (figure 7A), probiotic-treated mice (figure 7B), and maltodextrin-treated mice (figure 7C) showed normal morphology and few apoptotic cells. In contrast, colon sections from mice infected with C. rodentium (figure 7D) demonstrated cellular damage, cell exfoliation, and an increased number of TUNEL-positive cells in sloughed mucosa as well as in the crypts. Scattered throughout was nuclear dust, which is indicative of late stages of apoptosis [32, 33]. Pretreatment with the vehicle control, maltodextrin, resulted in increased numbers of TUNEL-positive cells in the colonic crypts (37.25 ± 6.4 TUNEL-positive cells/10 colonic crypts), compared with those in untreated control mice (3.5 ± 1.1 TUNEL-positive cells/10 colonic crypts) (P < .01, ANOVA) (figure 7E). Pretreatment with probiotics maintained colonic cellular integrity in C. rodentium–infected mice (figure 7F). There was no difference in the number of apoptotic colonic epithelial cells in tissues from untreated control mice (3.5 ± 1.1 TUNEL-positive cells/10 colonic crypts) and tissues from mice that received probiotics before C. rodentium infection (26.0 ± 4.8 TUNEL-positive cells/10 colonic crypts) (P > .05, ANOVA). In addition, staining for active caspase-3 produced a comparable pattern (figure 8).

Immunomodulatory effects of probiotics. Antiinflammatory cytokine IL-10 levels were higher in C. rodentium–infected mice (264.2 ± 39.2 pg/mL) than in uninfected mice (66.4 ± 28.2 pg/mL) (P = .003) (figure 9A). Treatment with probiotics in uninfected mice also increased IL-10 levels (1209.2 ± 145.3 pg/mL; P < .01, ANOVA). The administration of probiotics to

**Figure 5.** Reduction in *Citrobacter rodentium*–induced inflammatory cell infiltration into the lamina propria after pretreatment with probiotics. *C. rodentium* infection caused a marked increase in the cellular infiltrate in the lamina propria of C57BL/6 mice, compared with mice treated with probiotics alone. Probiotic pretreatment before challenge with *C. rodentium* resulted in a significant decrease in the lamina propria inflammation toward control levels. Results are mean ± SE. *P < .05 vs. probiotic treated mice, **P < .05 vs. C. rodentium.
Figure 6. Transmission electron photomicrographs of mouse colon. A, Morphological characteristics of the colon in mice treated with probiotics alone. Cellular organelles have normal morphological characteristics, with normal nucleus, intact microvilli, and luminal fusiform bacteria in the overlying mucus layer. B, Damage caused by Citrobacter rodentium infection. Infection caused mucosal damage with abnormal cellular morphology and the disruption of microvilli (short arrow). Chromatin condensation (long arrow) in the nucleus provides evidence of apoptosis. C, Fusiform bacteria, which have been internalized after C. rodentium infection (arrowhead) and subnuclear membrane vacuolarizations in the nuclei of most epithelial cells (dotted arrow). D, Decrease in the amount of bacterial internalization by pretreatment with probiotics, restored goblet-cell (G) formation, and increased mucin secretion. Intact apical microvilli appear to be normal, but chromatin condensation in the nucleus is still apparent. Approximate original magnifications: A, ×3000; B and D, ×5000; and C, ×7000.

Infected mice also resulted in increased IL-10 levels (685.5 ± 158.4 pg/mL), compared with those in mice infected with C. rodentium (264.2 ± 39.2 pg/mL) (P = .002, Student’s t test). Unexpectedly, pretreatment of mice with maltodextrin before infection with C. rodentium also increased IL-10 production by splenocytes (1145 ± 186.5 pg/mL), compared with that in both mice infected with C. rodentium alone (264 ± 39.2 pg/mL) and those treated with maltodextrin alone (16.2 ± 5.2 pg/mL) (P < .001, ANOVA).

Interferon (IFN)-γ production was increased in splenocytes from C. rodentium–infected mice (534.4 ± 25.7 pg/mL), compared with that in uninfected mice (25 ± 8.2 pg/mL) and uninfected mice that were given probiotics (99.4 ± 39.6 pg/mL) (P < .001, ANOVA) (figure 9B). Pretreatment with probiotics did not reduce C. rodentium–induced IFN-γ production (555.8 ± 39.2 pg/mL). There were no changes in IL-5 production (i.e., a Th2 cellular immune response) in splenocytes infected with C. rodentium versus sham-infected control mice (figure 9C).

DISCUSSION

The present study has demonstrated that probiotics maintain colonic integrity and reduce both epithelial-cell hyperplasia and inflammatory-cell infiltration into the lamina propria in the colon of C. rodentium–infected mice. When they are ingested in sufficient amounts [34], probiotics have the ability to transiently colonize the gut of mice [35] and to modulate the host microenvironment to provide beneficial effects [25, 36]. Recent clinical trials have demonstrated this benefit in a variety of intestinal disorders, such as pouchitis, Crohn disease, and trav-
Figure 7. Nos. of apoptotic [TUNEL-positive stained] epithelial cells increase in colonic tissue from mice infected with *Citrobacter rodentium*. TUNEL staining (brown) (long arrows) of colonic mucosa from control mice (A), probiotic-treated mice (B), and maltodextrin-treated mice (C) show similar morphological characteristics and comparable nos. of apoptotic cells. In contrast, *C. rodentium* infection caused exfoliation of epithelial cells into the lumen (arrowhead), which includes apoptotic cells. Positively stained epithelial cells are also apparent at the base of the colonic crypt, and evidence of nuclear dust (i.e., apoptotic cell fragments) is distributed throughout the crypts (D). Colonic tissue from mice treated with maltodextrin (E) before challenge with *C. rodentium* infection shows TUNEL-stained positive cells primarily on the apical surface of the colonic mucosa. Fewer nos. of positively stained epithelial cells were observed when mice were pretreated with probiotics before challenge with *C. rodentium* infection (F). Approximate original magnification, ×200.

...er's diarrhea [37]. Although there is increasing evidence of the clinical benefits of probiotics, their precise mechanisms of action remain largely unknown [38–40].

Previous in vitro studies have indicated that increased cellular permeability and the formation of attaching-effacing lesions can be attenuated with probiotic pretreatment [41]. Resta-Le- ner and Barrett [42] demonstrated that a probiotic mixture consisting of *Streptococcus thermophilus* and *L. acidophilus* prevents the adverse effects of enteroinvasive *E. coli* on host epithelial cells. Other researchers have shown that specific pro-
Figure 8. Decreased no. of active caspase-3–positive colonic epithelial cells after probiotic pretreatment. Colonic sections from control untreated mice (A), probiotic-treated mice (B), and maltodextrin-treated mice (C) show similar morphological characteristics and comparable positive staining for caspase-3 activation (brown) (long arrows). In contrast, Citrobacter rodentium infection caused exfoliation of epithelial cells into the lumen (arrowheads), which includes apoptotic cells. Positively stained colonic epithelial cells are also apparent at the base of the colonic crypt (D). In addition, colonic tissue treated with maltodextrin (E) shows high nos. of apoptotic epithelial cells, compared with colonic tissue from untreated control mice. Pretreatment with probiotics before challenge with C. rodentium (F) resulted in fewer nos. of positively stained colonocytes. Approximate original magnification, ×200.
Figure 9. Increased interleukin (IL–10) secretion in splenocytes with probiotic treatment of mice. Interferon (IFN)–γ levels increased in mice with Citrobacter rodentium infection and remained elevated in the presence of probiotics (B). Increased in the levels of IL–10 were observed in probiotic-treated mice and those pretreated with maltodextrin before C. rodentium infection (A). All treated mice had IL–5 levels similar to those of control mice. Vehicle control and maltodextrin had no effect on IFN–γ, IL–10, or IL–5 levels in all mice (C). Results are mean ± SE. *P < .003 vs. control, **P = .002 vs. C. rodentium.

Probiotics have bactericidal effects against pathogenic bacteria [43]. Probiotics can directly prevent the growth of pathogenic bacteria [22, 44]. Previous in vitro studies with the specific Lactobacillus mixtures that we used have also shown beneficial effects against pathogenic bacteria [45]. These Lactobacillus species adhere to host epithelial cells and competitively exclude bacterial enteropathogens. The probiotic treatment of epithelial cells prevents rearrangements of host cytoskeletal proteins and thereby prevents attaching-effacing lesions after EPEC and EHEC infection [45].

The results of our in vitro studies have demonstrated that the probiotic mixture elicits direct inhibitory effects on the growth of C. rodentium. Bactericidal effects were shown with the inhibition of C. rodentium when culture supernatants of probiotics were used, which implicates the presence of secreted factors in the culture supernatants.

The present study has demonstrated the effects of C. rodentium infection on barrier function to be similar to those observed in response to exposure to EPEC and EHEC [9, 11, 24]. Changes in mucosal barrier function due to C. rodentium infection have also been observed in vivo [46, 47].

A recent study by Wiles et al. [48] demonstrated that both viable bacterial colony-forming units and bioluminescence (a technique used to monitor the in vivo growth dynamics of C. rodentium) peak 8–10 days after infection. Other investigators have also reported similar findings [15, 18]. Therefore, we used a time point of 9 days after infection to optimize both colonic bacterial colonization and mucosal inflammation after the orogastric challenge of mice with C. rodentium. This experimental approach allowed the effects of probiotics to be assessed at the maximum point of infection and disease severity, because there is a natural clearance of C. rodentium from the intestinal tract 10–14 days after infection [49].

Our results are in agreement with those of previous reports that demonstrated that C. rodentium causes mucosal damage and epithelial hyperplasia in the colon [50, 51]. Probiotics were shown to provide additional beneficial effects, such as preventing bacterial infiltration into the epithelial mucosa and increasing mucus production. This observation is supported by previous work that showed that lactobacilli adherent to host epithelial cells increase MUC3 secretion and decrease attaching-effacing bacterial adherence [52].

The induction of apoptosis by C. rodentium has previously been reported as early as 6 days after infection [15]. There are no known reports of studies that have investigated C. rodentium–induced apoptosis after 6 days of infection. This could be due to colonic mucosal damage, cell exfoliation, and the production of nuclear dust later during the course of the infection making it difficult to accurately measure changes in the number of apoptotic cells. The present study has shown that morphologically recognizable apoptotic colonic epithelial cells were detected with both TUNEL staining and caspase-3 analysis and has clearly demonstrated that the number of apoptotic colonocytes observed 9 days after infection was decreased by probiotic pretreatment.

Previous studies have demonstrated that a daily bolus administration of L. acidophilus strain NCFM reduces C. rodentium–induced colonic epithelial hyperplasia and modulates the immune system by decreasing levels of IFN–γ and IL–15 [53]. In addition, other studies have shown that levels of IL–10 in the human intestine increase after oral administration of a mix-

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ture of 8 different probiotics [54] but that the probiotics have no effect on IFN-γ secretion [55]. The present study in C. rodentium-infected mice has confirmed that probiotics have activity against bacterial enteric pathogens. This beneficial effect may be mediated through the ability of the probiotics to shift from a primarily proinflammatory Th1 cell response to a more balanced Th1/Th2 (regulatory) host immune response.

In conclusion, by use of the mouse model of microbial-induced colonic epithelial-cell hyperplasia, we have demonstrated that probiotics are able to ameliorate the sequelae of C. rodentium infection. These results also confirm that this mixture of live probiotics does not adversely affect the health of mice [35] and emphasize that probiotics alter host responses to microbial pathogens through multiple mechanisms of action, including the production of bactericidal substances, reduction of mucosal inflammation, maintenance of colonic mucosal integrity, and promotion of host antiinflammatory responses.

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**References**