Probiotic *Lactobacillus reuteri* Attenuates the Stressor-Enhanced Severity of *Citrobacter rodentium* Infection

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Stressor exposure has been shown to enhance host susceptibility and the severity of a plethora of illnesses, including gastrointestinal disease. In mice, susceptibility to *Citrobacter rodentium* has been shown to be dependent on host genetics as well as the composition of the intestinal microbiota, but the effects of stressor exposure on this gastrointestinal pathogen have not been elucidated fully. Previously, our lab showed that exposure to the prolonged-restraint stressor prior to a challenge with *C. rodentium* alters the intestinal microbiota community structure, including a reduction of beneficial genera such as *Lactobacillus*, which may contribute to stressor-enhanced *C. rodentium*-induced infectious colitis. To test the effects of stressor exposure on *C. rodentium* infection, we exposed resistant mice to a prolonged-restraint stressor concurrent with pathogen challenge. Exposure to prolonged restraint significantly enhanced *C. rodentium*-induced infectious colitis in resistant mice, as measured by increases in colonic histopathology, colonic inflammatory mediator gene production, and pathogen translocation from the colon to the spleen. It was further tested if the beneficial bacterium *Lactobacillus reuteri* could reduce the stressor-enhanced susceptibility to *C. rodentium*-induced infectious colitis. While *L. reuteri* treatment did not reduce all aspects of stressor-enhanced infectious colitis, it did significantly reduce pathogen translocation from the colon to the spleen. Taken together, these data demonstrate the deleterious effects that prolonged stressor exposure can have at the onset of a gastrointestinal infection by its ability to render a resistant mouse highly susceptible to *C. rodentium*. Probiotic treatment ameliorated the systemic manifestations of stress on colonic infection.

*Citrobacter rodentium*, along with enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively), belongs to the group of noninvasive pathogens collectively known as attaching and effacing (A/E) bacteria. In order to colonize its host, *C. rodentium*, along with its human homologs, must intimately attach to the intestinal epithelium. This interaction allows the pathogen to inject effector proteins into the host epithelial cell via a type III secretion system. These proteins, which include intimin, Tir, EspF, and Map, are located on the locus of enterocyte effacement pathogenicity island and are involved in the adherence to and effacement of the colonic epithelium, as well as the formation of actin-rich pedestals beneath the pathogen (1). Upon colonization of the colonic epithelium, A/E pathogens induce increases in leukocyte infiltration, inflammatory cytokine production, and structural changes to colonic tissue that are similar to those with EPEC and EHEC infection as well as both murine and human inflammatory bowel disease (1–3). Because mice are not naturally susceptible to intestinal EPEC or EHEC, murine infection with *C. rodentium* has become a widely used model of intestinal *E. coli* infection. *C. rodentium* does not normally cause disease in humans but has been shown to colonize all strains of mice. The disease can be either subclinical or fatal, depending on the host mouse strain (4). Strains that develop only subclinical symptoms, such as CD-1 and C57BL/6, are considered resistant to *C. rodentium*-induced infectious colitis, while mice that succumb to infection by *C. rodentium*, such as FVB/N and C3H/HeJ mice, are deemed susceptible (1, 4, 5). While it is recognized that host genetics contributes to enhanced susceptibility (4, 5), recent data indicate that the colonic microbiota also modulates host susceptibility to *C. rodentium*, in part by altering mucosal immunity (6–8). Antibiotic treatment can disrupt the natural balance of the microbiota, which can leave mice more susceptible to *C. rodentium* infection (9). Conversely, supplementation with beneficial bacteria can reduce susceptibility to *C. rodentium* infection (10–13).

Animal stressors are useful for our understanding of the impact of the host stress response on infectious diseases. Our laboratory has shown that exposure to laboratory stressors, including the most commonly used experimental stressor in biomedical and biobehavioral research, namely, physical restraint (14), significantly changes the structure of the colonic microbiota and reduces the levels of beneficial members of the genus *Lactobacillus* (15–18). Because lactobacilli can help to regulate mucosal inflammation and mediate resistance/susceptibility to *C. rodentium* (10–13, 19, 20), we hypothesized that exposing resistant CD-1 mice to a prolonged-restraint stressor during challenge with *C. rodentium* would significantly increase disease pathology.

It has long been known that exposure to psychological stressors can exacerbate both infectious and noninfectious diseases, and gastrointestinal (GI) diseases such as Crohn’s disease, ulcerative colitis, and enteric infectious disease are often exacerbated during stressful periods (21, 22). While the mechanisms by which stressor...
exposure exacerbates GI diseases are still under investigation, disruptions of gut epithelial permeability are likely involved. The GI tract is lined by a single layer of epithelial cells that keeps the interior of the body separated from the contents of the gut. During stressful life events, the gut can become inflamed (23), and the tight junctions that help to control what is transported across the epithelial layer are loosened, leading to a “leaky gut.” The loosening of tight junctions, chiefly by the proinflammatory cytokine tumor necrosis factor alpha (TNF-α), allows the contents of the gut, including commensal microbes, to pass through the epithelial layer and enter into the lamina propria.

The penetration of microbes into the lamina propria results in a stronger inflammatory response, in part through activation of mucosal immune cells (24). Thus, strategies to reinforce the epithelial barrier and suppress mucosal immune activity could be beneficial in preventing stressor-induced exacerbations of GI diseases. One potentially useful strategy involves the administration of probiotic microbes, such as *Lactobacillus reuteri*. The Gram-positive bacterium *L. reuteri* can be found in both humans and laboratory animals, including mice. Many strains of *L. reuteri* are currently being used as probiotics, because research has shown that *L. reuteri* can suppress inflammation and immune cell activity (25–28). Moreover, *L. reuteri* has been shown to reduce experimental colitis and prevent experiment-induced intestinal permeability in rats (29, 30). Because stressor exposure reduces the levels of commensal *L. reuteri* found in the murine colon (J. Galley and M. T. Bailey, unpublished observations), this study tested the hypothesis that stressor exposure would increase the severity of *C. rodentium*-induced colonic pathology in a resistant mouse strain. This study also tested the corollary hypothesis that treating the mice with *L. reuteri* would prevent any stressor-induced increases in disease severity.

**MATERIALS AND METHODS**

**Mice.** Outbred, male CD-1 mice aged 6 to 8 weeks were purchased from Charles River Laboratories (Wilmington, MA) and housed at 3 mice per cage, unless otherwise indicated, in ventilated polycarbonate cages (32 cm × 18 cm × 15 cm). For all experiments, mice were allowed to acclimate to their surroundings for 1 week prior to experimentation. Lights were maintained on a 12-h on, 12-h off cycle (lights on at 0600). Mice were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility and had free access to food and water except during experimental procedures. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University (protocol 2009A0235).

**Prolonged restraint.** Prolonged restraint was performed as previously described (18, 31). Briefly, mice were divided into the following three groups: home-cage control (HCC), food- and water-deprived (FWD), and prolonged-restraint (RST) groups. Restrainted mice were placed in well-ventilated 50-ml conical tubes at the beginning of their active cycle (i.e., at 1800) and were removed from the tubes the following morning at 0900. Because the stressor-exposed mice did not have access to food or water at the time of restraint, FWD mice had their food and water removed from their cages at the time of restraint but were allowed to move freely about their cages. Both RST and FWD mice were allowed free access to their food and water from 0900 to 1800. Mice were restrained or deprived of food and water each night for seven consecutive nights. Control mice were left undisturbed in their cages for the duration of the experiment.

**Infection and bacterial enumeration.** *Citrobacter rodentium* strain DBS120(pCRP1::Tn5) (32) was grown overnight in Difco Lennox broth (LB) at 37°C. Cultures were centrifuged, and the resulting pellet was re-suspended in sterile phosphate-buffered saline (PBS) to give a final concentration of 3 × 10⁷ CFU/ml. Mice were then challenged at 0900 on the day following the first night of restraint via oral gavage with 100 μl containing 3 × 10⁷ CFU and were food and water deprived for 2 h postchallenge. Mice in the RST group were restrained during the first 6 days postchallenge. The day of infection is referred to as day 0, and all data collected are referenced by the number of days postchallenge. Fecal shedding of *C. rodentium* was monitored on days 1, 3, 6, 12, and 24 postchallenge. Fresh stool was collected from individual mice by placing them in a sterile cage and collecting 1 or 2 stool pellets per mouse. The stool was then weighed, homogenized manually in 3 ml sterile PBS, and serially diluted at a ratio of 1:20. In order to enumerate *C. rodentium* only, each dilution was grown via the pour plate method in MacConkey agar supplemented with the antibiotic kanamycin (40 μg/ml) overnight at 37°C. At the time of sacrifice, spleens were flash frozen in liquid nitrogen. Spleens were then thawed, weighed, and suspended in 5 ml sterile PBS. Spleens were homogenized using a stomacher (medium speed for 1 min) and then serially diluted and plated in the same fashion as stool samples.

**Experiment 1 design.** The first set of experiments was designed to test the hypothesis that prolonged stressor exposure would exacerbate *C. rodentium*-induced infectious colitis. To test the effects of prolonged stressor exposure on the severity of *C. rodentium* infection, two replicate time course experiments were performed. In the first time course experiment, all mice were housed at 3 mice/cage/group (i.e., HCC, FWD, or RST). The mice in one cage for each group were sacrificed on days 1, 3, 6, 12, and 24 postchallenge. In the replicate time course experiment, all mice were housed at 5 mice/cage/group, with the exception of mice to be sacrificed on day 12 postchallenge, which were housed at 5 mice/cage/group. The mice in one cage for each group were sacrificed on days 0, 1, 3, 6, 12, and 24 postchallenge, for a grand total of 105 mice between the two time course experiments. There were no differences in any of the measurements taken from mice that were housed at either 3 or 5 per cage on day 12 postchallenge (analysis not shown). For each experiment, all mice were infected with *C. rodentium* immediately following the first night of stressor exposure, with the exception of mice sacrificed on day 0, as these mice were used as noninfected baseline controls for colonic gene expression analyses. Stressor exposure continued for 6 more days, after which mice were left alone in their cages throughout the remainder of the experiment. Each time point reflects the day postchallenge. Mice were sacrificed at the designated time points by CO₂ asphyxiation, and tissue samples were collected. Because there were no differences between the HCC and FWD control groups for any of the measures assessed, these groups were combined into one control group (CON).

**Probiotic administration.** *Lactobacillus reuteri* strain 23272 (American Type Culture Collection [ATCC]) was grown overnight in Difco Lactobacilli MRS broth at 37°C with 5% CO₂. Cultures were centrifuged, and the resulting pellets were resuspended in sterile PBS (vehicle [VEH]). Cultures were then added to the drinking water to give a final concentration of 5 × 10⁸ CFU/ml of drinking water. Mice received either probiotic or VEH in their water from 1 day prior to the first day of stressor exposure until the mice were sacrificed on day 12 postchallenge. Water supplemented with *L. reuteri* was monitored throughout the duration of the experiment to ensure viability. One milliliter of water was removed via a Lixit water bottle and serially diluted 1:20. Each dilution was grown on Difco Lactobacilli MRS agar at 37°C with 5% CO₂. Cultures were centrifuged, and the resulting pellets were resuspended in sterile PBS (vehicle [VEH]). The bacteria were then added to the drinking water to give a final concentration of 5 × 10⁸ CFU/ml of drinking water. Mice received either probiotic or VEH in their water from 1 day prior to the first day of stressor exposure until the mice were sacrificed on day 12 postchallenge. Water supplemented with *L. reuteri* was monitored throughout the duration of the experiment to ensure viability. One milliliter of water was removed via a Lixit water bottle and serially diluted 1:20. Each dilution was grown on Difco Lactobacilli MRS agar at 37°C with 5% CO₂. Cultures were centrifuged, and the resulting pellets were resuspended in sterile PBS (vehicle [VEH]).

**Experiment 2 design.** The second set of experiments was designed to test the hypothesis that probiotic intervention could prevent stressor-enhanced disease severity associated with *C. rodentium* challenge. Because our initial set of experiments demonstrated that the peak of *C. rodentium*-induced infectious colitis occurred on day 12 postchallenge, our second set of experiments was focused on day 12 postchallenge. Our hypothesis was tested over 3 replicate experiments involving 6 experimental groups (i.e., HCC/VEH, HCC/L. reuteri, FWD/VEH, FWD/L. reuteri, RST/VEH, and...
and RST/L. reuteri). Mice were housed at 3 mice/cage/group, for a total of 54 infected mice. Noninfected control mice were used as baseline control samples for real-time PCR analysis (4 cages of noninfected control mice housed at 3 mice/cage, for a total of 12 noninfected control mice). Stressor exposure and C. rodentium challenge were the same as in the first set of experiments, but mice were given probiotic L. reuteri in their water beginning 1 day prior to stressor exposure and continuing through day 12 postchallenge. Mice were sacrificed on day 12 postchallenge by CO2 asphyxiation, and tissue samples were collected. Because there were no differences between the HCC and FWD mice within treatment groups for any of the measures assessed in the second set of experiments, these groups were combined based on treatment condition (CON/VEH and CON/L. reuteri).

**Histopathology.** On days 0, 1, 3, 6, 12, and 24 postchallenge, colons were excised and cut in half longitudinally. One section was used for further analysis of colonic cytokines by real-time PCR, and the other was snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis of colonic histopathology. The severity of lesions in the colon was scored according to pre-defined criteria (33). Briefly, each colon was scored on 5 different categories: inflammation, dysplasia, hyperplasia, edema, and epithelial defects. Each category received a score of 0 to 4 in increments of 0.5, based on the degree of lesion severity, as follows: 0, absent; 1, mild; 2, moderate; 3, marked; and 4, severe. All 5 categories were added together to garner a total histologic colitis index score with a maximum value of 20.

**Semiquantitative real-time PCR.** Colons were harvested, and total RNA was isolated using a standard single-step isolation protocol (TRI-zol; Invitrogen). After isolation, total RNA was quantified spectrophotometrically. A reverse transcription reaction was performed to synthesize cDNA by use of an avian myeloblastosis virus (AMV) reverse transcriptase enzyme kit (Promega Corporation, Madison, WI). These samples then underwent real-time PCR in order to amplify the nucleotide sequences of interest. In all cases, the 18S rRNA gene was used as the housekeeping gene. The sequences of primers and probes used can be found in Table 1. SYBR green was used in place of a labeled probe sequence for exposure and samples for real-time PCR analysis (4 cages of noninfected control mice housed at 3 mice/cage, for a total of 12 noninfected control mice). Stressor exposure and C. rodentium challenge were the same as in the first set of experiments, but mice were given probiotic L. reuteri in their water beginning 1 day prior to stressor exposure and continuing through day 12 postchallenge. Mice were sacrificed on day 12 postchallenge by CO2 asphyxiation, and tissue samples were collected. Because there were no differences between the HCC and FWD mice within treatment groups for any of the measures assessed in the second set of experiments, these groups were combined based on treatment condition (CON/VEH and CON/L. reuteri).

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<table>
<thead>
<tr>
<th>Primer or probe</th>
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<th>Sequence</th>
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<tr>
<td>CCL2</td>
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<td>TTGGCCTAGCCAGATGCA</td>
</tr>
<tr>
<td>CCL2</td>
<td>Reverse</td>
<td>CCTACTGTTGGATCTGTGCC</td>
</tr>
<tr>
<td>TNF-α</td>
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<tr>
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<td>Reverse</td>
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</tr>
<tr>
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<tr>
<td>Claudin 5</td>
<td>Reverse</td>
<td>TTATAGATGCTTCCCTGCTGTA</td>
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*Primer or probes were purchased from Invitrogen (Carlsbad, CA), and probes were purchased from Applied Biosystems (Foster City, CA).*
hallmark of *C. rodentium* infection, was significantly increased on day 12 postchallenge in the stressor-exposed group *P* < 0.0001 (Fig. 2A). Total colonic histopathology, which is the combination of scores given to inflammation, edema, hyperplasia, dysplasia, and epithelial defects, was also significantly increased by prolonged restraint *F*(2, 45) = 8.2; *P* = 0.001 (Fig. 1C). Control colons had a relatively normal appearance, with small numbers of lymphocytes infiltrating the mucosa (Fig. 1D). However, stressor-increased histopathology was evidenced by mucosal thickening, regional ulcerations in the surface epithelium, and a significant inflammatory cell infiltrate that extended from the mucosa to the edematous submucosa (Fig. 1E).

**Prolonged restraint enhances inflammatory mediator gene expression in the colons of infected mice.** Colonic inflammatory mediator mRNA expression (i.e., CCL2, TNF-α, and inducible NO synthase [iNOS] mRNAs) was assessed using real-time PCR. Colonic CCL2 mRNA expression was significantly increased by stressor exposure throughout the 24-day experiment compared to the control level *F*(5, 90) = 4.5; *P* = 0.001 (Fig. 2A). Mice exposed to prolonged restraint also had enhanced colonic TNF-α mRNA expression compared to nonstressed controls *F*(5, 93) = 9.2; *P* < 0.001 (Fig. 2B). Similar to that of CCL2 and TNF-α, colonic iNOS gene expression was significantly increased throughout the 24-day experiment compared to colons of control mice *F*(5, 93) = 45.3; *P* < 0.0001 (Fig. 2C). The most evident increases in colonic CCL2, TNF-α, and iNOS mRNA expression occurred on day 12 postchallenge.

**Prolonged restraint causes significant changes in spleens of infected animals.** One of the main characteristics of prolonged restraint is a reduction in spleen mass (37), which was observed on days 1, 3, and 6 postchallenge (i.e., after 2, 5, and 7 days of restraint) in stressor-exposed mice (Fig. 3A). This reduction was not evident throughout the experiment, but there were significant changes in spleen mass across the 24-day experiment *F*(4, 76) = 20.1; *P* < 0.001. To determine whether the unexpected increase in spleen mass on day 12 postchallenge in stressor-exposed mice was associated with an increased escape of *C. rodentium* from the colon and translocation to the spleen, we set out to determine if *C. rodentium* could be cultured from the spleen. On days 0, 1, 3, 6, and 24 postchallenge, there were no countable colonies in the CON and RST groups (data not shown). However, on day 12 postchallenge, there was an increase in the likelihood (88% versus 25%) of observing *C. rodentium* in the spleens of stressor-exposed mice *X*²(1) = 8.21; *P* < 0.005 (Fig. 3B). Not only was there an increase in the likelihood of detecting *C. rodentium* in the spleens of stressor-exposed mice, but there was also an enhanced pathogen burden in those mice found to have *C. rodentium* in the spleen *F*(1, 22) = 10.0; *P* < 0.01 (Fig. 3C).

**Probiotic intervention does not reduce RST-enhanced pathogen colonization or colonic histopathology.** As with the previous experiment, stressor exposure significantly increased fecal *C. rodentium* levels on day 12 postchallenge *F*(1, 50) = 17.2; *P* < 0.0001 (Fig. 4A). There were no changes in colonization between VEH-treated and *L. reuteri*-treated groups. Treatment
with L. reuteri also had no effect on the stressor-induced increase in colonic histopathology. Colonic mass on day 12 postchallenge was significantly increased in both the VEH- and L. reuteri-treated stressor-exposed mice compared to control mice [$F(1, 49) = 24; P < 0.001$] (Fig. 4B). Total colonic histopathology was also increased in the RST mice compared to the CON mice [$F(1, 28) = 9.4; P = 0.005$] (Fig. 4C). In no case did treatment with L. reuteri affect colonic histopathology; histopathology was similar in VEH-treated and L. reuteri-treated mice. No lesions were observed in mice from either control group (Fig. 4D and E). Stressor-enhanced histopathology was evidenced by lesions containing inflammation, epithelial defects, hyperplasia, and dysplasia. Neutrophilic inflammation extended from the mucosa to the submucosa and was frequently associated with epithelial erosion and ulceration (Fig. 4F and G).

**Probiotic intervention reduces stressor-enhanced colonic gene expression.** Colonic inflammatory mediator gene expression was previously shown to be increased on day 12 post-\textit{C. rodentium} challenge in the RST group compared to the control counterparts. Stressor exposure caused a significant increase in colonic CCL2 mRNA gene expression on day 12 postchallenge [$F(1, 49) = 22.4; P < 0.001$] (Fig. 5A). While \textit{L. reuteri} treatment reduced the mean level of CCL2 mRNA, the \textit{L. reuteri}-induced reduction was not large enough to be considered statistically significant. Similarly, colonic TNF-\textalpha gene expression also experienced a significant increase in stressor-exposed mice compared to nonstressed, infected controls [$F(1, 50) = 23.5; P < 0.001$] (Fig. 5B), with a nonsignificant reduction in \textit{L. reuteri}-treated, stressor-exposed mice. Stressor exposure also caused a significant increase in colonic iNOS gene expression [$F(1, 50) = 35.2; P < 0.001$] (Fig. 5C). This stressor-enhanced increase of colonic iNOS mRNA was unchanged by \textit{L. reuteri} treatment. The antimicrobial peptide \beta-defensin 3 was significantly reduced in the colons of \textit{C. rodentium}-challenged, stressor-exposed mice compared to infected, nonstressed, infected controls.
nonstressed controls \( [F(1, 30) = 6.5; P < 0.05] \) (Fig. 5D). This stressor-enhanced 5-fold decrease in \( \beta \)-defensin 3 gene expression in colons of VEH-treated mice was reversed by \( L. \text{reuteri} \) treatment. There was a significant reduction in colonic claudin 1 mRNA expression in stressor-exposed mice compared to nonstressed controls \( [F(1, 30) = 15.0; P = 0.001] \) (Fig. 5E), an effect which was unchanged by \( L. \text{reuteri} \) treatment. Claudin 5 was also significantly reduced in stressor-exposed mice compared to nonstressed controls \( [F(1, 29) = 6.0; P = 0.01] \) (Fig. 5F). The observed stressor-enhanced decrease in VEH-treated mice was reversed by \( L. \text{reuteri} \) treatment.

Probiotic intervention reduces stressor-enhanced pathogen dissemination to the spleen. On day 12 postchallenge, spleen mass was significantly changed by \( L. \text{reuteri} \) supplementation \( [F(1, 50) = 7.6; P < 0.01] \) (Fig. 6A). There was a significant increase in day 12 spleen mass in stressor-exposed, VEH-treated mice, and this was prevented by \( L. \text{reuteri} \) treatment. On day 12 postchallenge, there was a significant increase in the likelihood of discovering \( C. \text{rodentium} \) in stressor-exposed mice compared to nonstressed mice \( [\chi^2(1) = 4.75; P < 0.05] \) (Fig. 6B). While there were no significant changes in pathogen dissemination in VEH-treated and \( L. \text{reuteri} \)-treated RST groups, there was a 24% drop in pathogen prevalence in spleens from \( L. \text{reuteri} \)-treated, stressor-exposed mice (78% versus 54%). Pathogen burden was also significantly increased in stressor-exposed mice compared to nonstressed control mice \( [F(1, 47) = 6.1; P < 0.05] \) (Fig. 6C). This effect was diminished in \( L. \text{reuteri} \)-fed, stressor-exposed mice.

Probiotic intervention reduces anxiety-like behaviors and systemic IL-6. Exposure to prolonged restraint during \( C. \text{rodentium} \) challenge did not affect locomotion in a novel environment (Fig. 7A). However, there was a trend for an interaction between stressor exposure and probiotic treatment (i.e., \( L. \text{reuteri} \)) in that mice that were fed \( L. \text{reuteri} \) had a reduction in the amount of time spent in the center of the open field compared to stressor-exposed, VEH-treated mice \( [F(1, 49) = 3.30; P = 0.075] \) (Fig. 7B). Even

**FIG 4** Probiotic intervention with \( L. \text{reuteri} \) does not reduce stressor-enhanced pathogen colonization or colonic histopathology. Mice were restrained for 1 day and then orally challenged with \( C. \text{rodentium} \). Restraint continued for 6 days postchallenge, during which the drinking water was supplemented with either PBS vehicle (VEH) or \( L. \text{reuteri} \) beginning 1 day prior to restraint. (A) To determine the extent of colonization by \( C. \text{rodentium} \), stool samples were collected on day 12 postchallenge. Exposure to restraint stress significantly increased \( C. \text{rodentium} \) colonization compared to that in CON mice, regardless of treatment. *, ANOVA main effect for group (CON versus RST; \( P < 0.0001 \)). Sample sizes were as follows: \( n = 18 \) (CON/VEH), \( n = 18 \) (CON/\( L. \text{reuteri} \)), \( n = 9 \) (RST/VEH), and \( n = 9 \) (RST/\( L. \text{reuteri} \)). (B) Colonic tissue was removed on day 12 postchallenge and weighed without contents. Exposure to prolonged restraint significantly increased the weight of the colonic tissue compared to that of CON mice, regardless of probiotic intervention. *, ANOVA main effect for group (CON versus RST; \( P < 0.0001 \)). Sample sizes were as follows: \( n = 18 \) (CON/VEH), \( n = 17 \) (CON/\( L. \text{reuteri} \)), \( n = 9 \) (RST/VEH), and \( n = 9 \) (RST/\( L. \text{reuteri} \)). On day 12 postchallenge, colons were removed, fixed in formalin, and embedded in paraffin. Colons were sectioned and stained with hematoxylin and eosin in order to visualize and score the pathology present in each sample. (C) Stressor exposure enhanced colonic histopathology in both VEH- and \( L. \text{reuteri} \)-treated mice. *, ANOVA main effect for group (CON versus RST; \( P = 0.005 \)). Sample sizes were as follows: \( n = 11 \) (CON/VEH), \( n = 12 \) (CON/\( L. \text{reuteri} \)), \( n = 5 \) (RST/VEH), and \( n = 4 \) (RST/\( L. \text{reuteri} \)). (D) Representative image of H&E-stained colon from a CON/VEH mouse. (E) Representative image of H&E-stained colon from a CON/\( L. \text{reuteri} \) mouse. (F) Representative image of H&E-stained colon from an RST/VEH mouse. (G) Representative image of H&E-stained colon from an RST/\( L. \text{reuteri} \) mouse. For panels F and G, an asterisk marks an area with significant inflammatory cell infiltrate, “X” marks an area with surface epithelial erosions, and “M” marks dysplasia due to disorganized mucosal glands.
FIG 5 Probiotic intervention reduces stressor-enhanced colonic gene expression during the peak of infection. Mice were restrained for 1 day and then orally challenged with C. rodentium. Restraint continued for 6 days postchallenge, during which the drinking water was supplemented with either VEH or L. reuteri, beginning 1 day prior to restraint. Colonos were processed in order to quantify gene expression by real-time PCR. (A) Stressor exposure significantly increased gene expression of CCL2, regardless of treatment. *, ANOVA main effect for group (CON versus RST; P < 0.001). Sample sizes were as follows: n = 17 (CON/VEH), n = 18 (CON/L. reuteri), n = 9 (RST/VEH), and n = 9 (RST/L. reuteri). (B) Stressor exposure also significantly enhanced TNF-α gene expression in both VEH- and L. reuteri-treated mice. *, ANOVA main effect for group (CON versus RST; P < 0.001). Sample sizes were as follows: n = 18 (CON/VEH), n = 18 (CON/L. reuteri), n = 9 (RST/VEH), and n = 9 (RST/L. reuteri). (C) Stressor exposure significantly increased colonic iNOS gene expression in both VEH- and L. reuteri-treated mice. *, ANOVA main effect for group (CON versus RST; P < 0.001). Sample sizes were as follows: n = 18 (CON/VEH), n = 18 (CON/L. reuteri), n = 9 (RST/VEH), and n = 9 (RST/L. reuteri). (D) Stressor exposure significantly reduced β-defensin 3 gene expression only in VEH-treated mice. *, P < 0.05 between CON/VEH and RST/VEH groups as assessed by Student’s t test with the Bonferroni correction factor as a post hoc test. Sample sizes were as follows: n = 12 (CON/VEH), n = 12 (CON/L. reuteri), n = 6 (RST/VEH), and n = 4 (RST/L. reuteri). (E) Claudin 1 was also significantly reduced in RST mice, regardless of the treatment group. *, ANOVA main effect for group (CON versus RST; P = 0.001). Sample sizes were as follows: n = 12 (CON/VEH), n = 12 (CON/L. reuteri), n = 6 (RST/VEH), and n = 4 (RST/L. reuteri). (F) Claudin 5 was significantly reduced by RST only in VEH-treated mice. *, P < 0.05 between CON/VEH and RST/VEH groups. Student’s t test with the Bonferroni correction factor was used as a post hoc test. Sample sizes were as follows: n = 12 (CON/VEH), n = 12 (CON/L. reuteri), n = 5 (RST/VEH), and n = 4 (RST/L. reuteri).

though this interaction was not quite statistically significant using a two-factor ANOVA, we tested our a priori hypothesis that VEH-treated mice exposed to the stressor would spend less time in the center of the open field than VEH-treated controls. As predicted, stressor exposure significantly reduced the time in the center of the open field in VEH-treated, stressor-exposed mice compared to VEH-treated control mice [F(1, 34) = 10.2; P < 0.01] (Fig. 7C). Lactobacillus reuteri treatment did not change serum IL-1β levels in either control or stressor-exposed groups. However, there were changes in serum levels of IL-6 [F(1, 36) = 7.3; P = 0.01] (Fig. 7D). On day 12 postchallenge, there was a significant increase in circulating IL-6 in VEH-treated, stressor-exposed mice compared to VEH-treated control mice. This stressor-enhanced increase in circulating IL-6 was blocked in stressor-exposed mice that were treated with L. reuteri. There were no significant changes in serum levels of IL-12p70, IFN-γ, TNF-α, IL-10, or IL-2 between CON or RST mice in either treatment group (Table 2).

**DISCUSSION**

The results of this study confirm previous reports that outbred CD-1 mice do not develop severe colitis when challenged with C. rodentium (1, 38, 39). Low levels of the pathogen were evident in the stool of nonstressed control mice orally challenged with 300 million CFU of C. rodentium, and these control mice developed very low levels of colitis. However, simply exposing these mice to a prolonged-restraint stressor during pathogen challenge was sufficient to significantly increase disease severity. Stressor exposure significantly increased C. rodentium levels in the colon and also the expression of pathogen-induced chemokines and cytokines. These inflammatory mediators aid in the recruitment of immune cells to help fight infection and also contribute to the eradication of pathogens. However, overexpression of these genes can contribute to colonic tissue damage and immunopathology. Even though all mice were orally challenged with C. rodentium, chemokine and cytokine mRNA levels in the colon of mice exposed to the stressor during challenge with C. rodentium were 5 to 250 times higher than the levels found in nonstressed control mice. One of these chemokines, namely, CCL2, is known to recruit inflammatory monocytes to the colon (40, 41), and inflammatory...
monocytes are prolific producers of TNF-α and iNOS (41). Thus, it is possible that overexpression of CCL2 recruits a larger number of inflammatory monocytes to the colon, ultimately leading to it is possible that overexpression of CCL2 recruits a larger number of inflammatory monocytes to the colon, ultimately leading to

**FIG 6** Probiotic intervention reduces stressor-enhanced pathogen translocation to the spleen. Mice were restrained for 1 day and then orally challenged with C. rodentium. Restraint continued for 6 days postchallenge, during which the drinking water was supplemented with either VEH or L. reuteri, beginning 1 day prior to restraint. Spleens were removed, weighed, and processed in order to enumerate C. rodentium by the pour plate method. (A) Stressor exposure significantly increased the size of the spleen on day 12 postchallenge in VEH-treated mice compared to CON/VEH mice. This effect was blocked in stressor-exposed mice that received L. reuteri. ***, P < 0.00001 between CON/VEH and RST/VEH groups; *, P < 0.01 between RST/VEH and RST/L. reuteri groups. Student’s t test with the Bonferroni correction factor was used as a post hoc test. Sample sizes were as follows: n = 18 (CON/VEH), n = 18 (CON/L. reuteri), n = 9 (RST/VEH), and n = 9 (RST/L. reuteri). (B) Stressor exposure significantly enhanced the likelihood of detecting the pathogen in the spleen in both VEH- and L. reuteri-treated mice. *, P < 0.05 between CON and RST groups as assessed by chi-square (χ²) analysis. Sample sizes were as follows: n = 17 (CON/VEH), n = 17 (CON/L. reuteri), n = 8 (RST/VEH), and n = 9 (RST/L. reuteri). (C) Stressor exposure significantly increased pathogen loads in the spleens of mice, regardless of treatment. *, ANOVA main effect for group (CON versus RST; P < 0.05). Sample sizes were as follows: n = 17 (CON/VEH), n = 17 (CON/L. reuteri), n = 8 (RST/VEH), and n = 9 (RST/L. reuteri). The horizontal line in panel C represents the detection limit for C. rodentium.

**FIG 6** Probiotic intervention reduces stressor-enhanced pathogen translocation to the spleen. Mice were restrained for 1 day and then orally challenged with C. rodentium. Restraint continued for 6 days postchallenge, during which the drinking water was supplemented with either VEH or L. reuteri, beginning 1 day prior to restraint. Spleens were removed, weighed, and processed in order to enumerate C. rodentium by the pour plate method. (A) Stressor exposure significantly increased the size of the spleen on day 12 postchallenge in VEH-treated mice compared to CON/VEH mice. This effect was blocked in stressor-exposed mice that received L. reuteri. ***, P < 0.00001 between CON/VEH and RST/VEH groups; *, P < 0.01 between RST/VEH and RST/L. reuteri groups. Student’s t test with the Bonferroni correction factor was used as a post hoc test. Sample sizes were as follows: n = 18 (CON/VEH), n = 18 (CON/L. reuteri), n = 9 (RST/VEH), and n = 9 (RST/L. reuteri). (B) Stressor exposure significantly enhanced the likelihood of detecting the pathogen in the spleen in both VEH- and L. reuteri-treated mice. *, P < 0.05 between CON and RST groups as assessed by chi-square (χ²) analysis. Sample sizes were as follows: n = 17 (CON/VEH), n = 17 (CON/L. reuteri), n = 8 (RST/VEH), and n = 9 (RST/L. reuteri). (C) Stressor exposure significantly increased pathogen loads in the spleens of mice, regardless of treatment. *, ANOVA main effect for group (CON versus RST; P < 0.05). Sample sizes were as follows: n = 17 (CON/VEH), n = 17 (CON/L. reuteri), n = 8 (RST/VEH), and n = 9 (RST/L. reuteri). The horizontal line in panel C represents the detection limit for C. rodentium.

monocytes are proliferative producers of TNF-α and iNOS (41). Thus, it is possible that overexpression of CCL2 recruits a larger number of inflammatory monocytes to the colon, ultimately leading to higher TNF-α and iNOS levels. While this hypothesis has not yet been tested completely, the colonic histopathology is consistent with this rationale. The total colitis index, which incorporated a scoring of the infiltration of leukocytes, ranged from 0.5 to 7 for the control mice to 10 to 15 for the stressor-exposed mice. This colitis, which in stressor-exposed mice included epithelial erosion and ulceration, further demonstrates the impact that stressor exposure can have on even “nonsusceptible” hosts.

Susceptibility and resistance to C. rodentium are largely dependent upon host genetics, but studies have shown that intestinal microbes, including probiotics, can also affect disease severity. In a previous study, we demonstrated that exposure to a prolonged-restraint stressor was sufficient to significantly alter microbial communities in the cecum (18). More recent data also indicate that the colonic tissue-associated microbiota can be affected in mice exposed to prolonged restraint, with a reduction in the colonic tissue-associated microbiota can be affected in mice exposed to prolonged restraint, with a reduction in that the colonic tissue-associated microbiota can be affected in mice exposed to prolonged restraint, with a reduction in

Throughout the duration of the experiment, it was consistently noted that stressor-exposed mice that were treated with L. reuteri exhibited fewer signs of illness (e.g., cleaner fur and more active behavior) than VEH-treated, stressor-exposed mice. Because overt illness behaviors can signify that the pathogen has escaped the colon and translocated to systemic sites, such as the spleen, we further investigated whether C. rodentium could be detected at systemic sites and whether systemic signs of illness were evident. Exposure to the stressor during challenge with C. rodentium resulted in a significant increase in the likelihood of detecting C. rodentium in the spleen. This is important, because unlike invasive gastrointestinal pathogens, such as Salmonella species, C. rodentium does not have mechanisms to invade its host (1, 44). Citrobacter rodentium and its attaching and effacing E. coli relatives stay within the digestive tract, attached to the apical surface of the colonic epithelium, during infections in an immunocompetent host (1, 44). However, during infection, C. rodentium can disrupt the tight junctions found between intestinal epithelial cells via the injection of effector proteins through a type III secretion system into the host epithelial cell. Among the inserted proteins are E. coli secreted protein F (EspF) and mitochondrion-associated protein (Map), which have been implicated in the disruption of tight junctions via changes in the expression and location of two integral proteins involved in the formation of the tight junction, namely, claudin 5 and zonula occludins 1 (45, 46). The noninvasive nature of C. rodentium, coupled with the fact that the tight junctions between intestinal epithelial cells help to regulate what can and cannot pass through the intestinal epithelial layer, led us to hypothesize that our stressor-enhanced translocation of pathogen from the colon to the spleen may have been due to the loosening of the epithelial tight junctions. In our model, we observed significant reductions in gene expression of both claudin 1 and claudin 5 in the colon and translocated to systemic sites, such as the spleen, on October 23, 2017 by guest http://iai.asm.org/Downloaded from
tion in claudin 5, which we believe contributed to the reduction of pathogen translocation from the colon to the spleen.

The ability of L. reuteri to reduce the translocation of C. rodentium from the colon to the spleen, potentially through stabilization of tight junction proteins, suggested that L. reuteri treatment would prevent the systemic manifestations of stressor exposure during C. rodentium challenge. Indeed, mice exposed to the stressor during C. rodentium challenge had significantly higher levels of circulating IL-6 but lower levels of IL-1β. Pathogen challenge often leads to behavioral changes in mice, in large part through the actions of circulating cytokines on brain biology. Previous studies have shown that elevations in circulating IL-6 can lead to the development of illness behavior and anxiety-like behavior in mice (47). In our study, treating mice with L. reuteri ameliorated the stressor-enhanced reduction in the amount of time mice spent in the center of the open field, suggesting that L. reuteri reduces anxiety-like behavior in mice exposed to the prolonged restraint stressor during C. rodentium challenge. Taken together, our data indicate that treatment with L. reuteri can attenuate the stressor-induced increases in pathogen translocation from the colon to the spleen, elevations in circulating IL-6, and anxiety-like behavior. Future studies will determine whether these factors are causally related and will determine whether stabilization of the epithelial tight junctions is necessary for the beneficial effects of L. reuteri.

Our study demonstrates the adverse effects of prolonged stress exposure on gastrointestinal infection and disease progression.

**TABLE 2 Serum cytokine levels**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CON-VEH Mean concn (pg/ml) ± SE</th>
<th>CON-L. reuteri Mean concn (pg/ml) ± SE</th>
<th>RST-VEH Mean concn (pg/ml) ± SE</th>
<th>RST-L. reuteri Mean concn (pg/ml) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12p70</td>
<td>25.0 ± 2.9</td>
<td>21.5 ± 4.1</td>
<td>21.7 ± 6.8</td>
<td>14.0 ± 4.0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>9.6 ± 1.4</td>
<td>9.7 ± 2.4</td>
<td>4.7 ± 1.6</td>
<td>6.6 ± 2.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>65.6 ± 12.5</td>
<td>71.1 ± 12.5</td>
<td>46.2 ± 14.3</td>
<td>44.8 ± 10.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>140.5 ± 18.1</td>
<td>126.2 ± 23.4</td>
<td>140.6 ± 42.6</td>
<td>84.6 ± 20.7</td>
</tr>
<tr>
<td>IL-2</td>
<td>4.6 ± 0.7</td>
<td>6.8 ± 1.7</td>
<td>3.3 ± 0.9</td>
<td>3.8 ± 1.1</td>
</tr>
</tbody>
</table>

* Cytokine levels were determined by Bio-Plex assay.

**FIG 7** Probiotic intervention reduces stressor-enhanced anxiety-like behavior and significantly reduces circulating IL-6 at the peak of infection. Mice were restrained for 1 day and then orally challenged with C. rodentium. Restraint continued for 6 days postchallenge, during which the drinking water was supplemented with either VEH or L. reuteri, beginning 1 day prior to restraint. Prior to sacrifice, mice were tested by the open field test and recorded for 5 min. Locomotion was quantified by the total number of lines crossed and the amount of time spent in the center of the open field. (A) There were no changes in the total number of lines crossed in either stressor-exposed group. Sample sizes were as follows: n = 12 (CON/VEH), n = 12 (CON/L. reuteri), n = 6 (RST/VEH), and n = 4 (RST/L. reuteri). (B) There was an increase in anxiety-like behavior, as evidenced by a reduction in the time spent in the center of the open field, in VEH-treated, stressor-exposed mice, which was restored with L. reuteri treatment. *, P < 0.05 between CON/VEH and RST/VEH groups as assessed by Student’s t test with the Bonferroni correction factor as a post hoc test. Sample sizes were as follows: n = 18 (CON/VEH), n = 18 (CON/L. reuteri), n = 9 (RST/VEH), and n = 9 (RST/L. reuteri). Cytokine levels were determined by a Bio-Plex assay. (C) Circulating IL-1β was reduced by stressor exposure on day 12 postchallenge, and L. reuteri failed to modulate this reduction, *, ANOVA main effect for group (CON versus RST; P = 0.01). Sample sizes were as follows: n = 13 (CON/VEH), n = 11 (CON/L. reuteri), n = 7 (RST/VEH), and n = 7 (RST/L. reuteri). (D) Circulating IL-6, however, was significantly increased by stressor exposure in VEH-treated mice on day 12 postchallenge. Probiotic intervention with L. reuteri reduced stressor-enhanced circulating IL-6 levels. *, P < 0.001 between CON/VEH and RST/VEH groups. Student’s t test with the Bonferroni correction factor was used as a post hoc test. Sample sizes were as follows: n = 14 (CON/VEH), n = 13 (CON/L. reuteri), n = 7 (RST/VEH), and n = 6 (RST/L. reuteri).
and suggests that probiotics may be beneficial for some, but not all, of the stressor effects. This may be particularly important for diseases such as postinfectious irritable bowel syndrome (PI-IBS), where infection severity and stressor exposure are both known to be predisposing factors (48). Understanding the mechanisms by which probiotics can affect gastrointestinal diseases and the conditions under which they have beneficial effects will facilitate the rational design and use of probiotic microbes.

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