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Probiotic *Lactobacillus reuteri* Ameliorates Disease Due to Enterohemorrhagic *Escherichia coli* in Germfree Mice▽

Kathryn A. Eaton,[1]* Alexander Honkala,[1] Thomas A. Auchtung,[2] and Robert A. Britton[2]

Unit for Laboratory Animal Medicine and Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0614,[1] and Microbial Genomics Laboratory, Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824[2]

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Strains of enterohemorrhagic *Escherichia coli* (EHEC) are a group of Shiga toxin-producing food-borne pathogens that cause severe hemorrhagic colitis and can lead to hemolytic-uremic syndrome (HUS), a life-threatening condition that principally affects children and for which there is no effective treatment. We used a germfree mouse model of renal and enteric disease due to EHEC to determine if probiotic *Lactobacillus reuteri* ATCC PTA 6475 is effective in suppressing disease symptoms caused by EHEC. When germfree Swiss Webster mice are monocolonized with EHEC, they develop disease characterized by weight loss, cecal luminal fluid accumulation, and renal tubular necrosis. When *L. reuteri* was administered 1 day prior to EHEC challenge and every other day thereafter, EHEC colonization was suppressed and mice were significantly protected from the manifestations of disease. Protection from disease did not require the induction of the antimicrobial compound reuterin in *L. reuteri* prior to treatment. The twice-daily administration of *L. reuteri* appeared more effective than every-other-day administration. These data indicated that *L. reuteri* partially protects mice from disease manifestations of EHEC.

Strains of enterohemorrhagic *Escherichia coli* (EHEC) are food-borne bacterial pathogens of humans that are normal inhabitants of the ruminant intestine (4). These organisms often are transmitted to humans in undercooked meat but also have caused outbreaks associated with contaminated vegetables (6, 25), fruit juices (8), and drinking water (28). Disease symptoms include watery or bloody diarrhea and can be severe enough to cause hospitalization and even death (18). In addition, some affected children (and rarely adults) develop hemolytic-uremic syndrome (HUS), a rapidly progressive and sometimes fatal form of renal failure that is attributed to the expression of Shiga toxins (Stx) (1, 18). HUS is a rare complication of disease due to EHEC, but its importance is amplified by its severity, tendency to affect young children, and the absence of any specific treatment or preventative measure (3). Thus, novel control and treatment therapies are needed.

Recently, several laboratories have explored the role of probiotic bacteria in the control and treatment of gastrointestinal diseases (20, 29), including EHEC (2, 5, 10, 11, 14, 15, 19, 23, 31, 32, 37). Probiotic bacteria are live microbes that, when consumed, confer a beneficial effect on the host. Their mechanism of action is not well understood, but in some cases specific probiotic organisms have been shown to reduce colonization by pathogens (20, 23), suppress toxin or other virulence factor production by pathogens (2, 11, 24, 32), or contribute to host defense mechanisms (20). *L. reuteri* is one of a few *Lactobacillus* species that has been shown to be indigenous to the human intestine and also is found in the intestinal tracts of several animal species, including the mouse (26, 34). *L. reuteri* ATCC 55730 is a commercial probiotic strain that survives in the human intestine (34) and has beneficial effects in clinical trials of infant diarrhea, colic, and IgE-dependent eczema (29, 30, 33, 36). *L. reuteri* was also shown to be effective in protecting interleukin-10 (IL-10)-deficient mice against spontaneous colitis (22).

Several potential mechanisms may confer the protective effects observed when *L. reuteri* is ingested. First, multiple strains of *L. reuteri* secrete a potent anti-inflammatory activity that reduces the expression of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) by greater than 90% in cultured activated macrophages (17, 21). Second, *L. reuteri* produces an antimicrobial compound, reuterin, which is bactericidal against many bacteria, including strains of EHEC (7). Reuterin (also known as 3-hydroxypropionaldehyde [3-HPA]) is an intermediate in the metabolism of glycerol to 1,3-propanediol, which allows the cell to regenerate NAD^+ during carbohydrate metabolism (35). It has potent antibacterial, antiviral, and antifungal activity, but lactic acid bacteria, including *L. reuteri*, are much more resistant to reuterin than are enteric pathogens, such as *E. coli* (7).

The goal of this study was to use a germfree mouse model of disease to determine if *L. reuteri* could be protective against either colonization by EHEC or disease caused by EHEC. Unlike specific-pathogen-free mice, germfree mice are highly susceptible to colonization by pathogenic EHEC of human origin and develop Shiga toxin (Stx)-dependent acute renal disease with features that resemble HUS in human children (9). Mice are susceptible to as few as 100 infectious organisms, and within 5 days the lower bowel is heavily colonized by up to 10^{11} organisms/g (9). EHEC strains that produce Stx2 cause a rapidly progressive, sometimes fatal disease that is characterized by increased cecal luminal fluid accumulation and acute kidney failure with renal tubular necrosis and glomerular fibrin thrombosis. Disease is Stx2 dependent, as indicated by the

* Corresponding author. Mailing address: Unit for Laboratory Animal Medicine, 018 Animal Research Facility, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0614. Phone: (734) 936-3883. Fax: (734) 936-3235. E-mail: kateaton@umich.edu.

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TABLE 1. Number of mice in each treatment group

Week	No. uninfected	No. with EHEC alone	No. with *L. reuteri* alone	No. with EHEC and *L. reuteri* by inoculation type	
				Every other day[a]	Twice daily
1	14	22	5	10 (6)	7
3	ND[b]	7	ND[b]	27 (19)	ND[b]

[a] The numbers in parentheses are the numbers of mice given inocula with reuterin induced (see the text).
[b] ND, not done.

failure of Stx2-deficient mutants to cause disease (9). Because both EHEC and *L. reuteri* colonize mice well and EHEC causes HUS-like renal disease in germfree mice, we used this model to determine if *L. reuteri* can protect mice against disease due to EHEC.

MATERIALS AND METHODS

Bacterial strains and culture conditions. EHEC strain 86-24 originally was isolated in association with an outbreak in humans (13) and causes disease in germfree mice (9). This strain produces Stx2. *L. reuteri* strain ATCC PTA 6475 is a human probiotic isolate that produces reuterin and suppresses TNF-α production by activated macrophages *in vitro* (17, 21). It also has endogenous kanamycin resistance, facilitating its recovery from cocolonized mice. *E. coli* 86-24 was cultured on sorbitol MacConkey (SMAC) agar or trypticase soy agar (TSA) with 5% sheep blood (blood agar). For inoculation, single colonies were transferred into Luria broth (LB) and incubated with shaking until the optical density at 600 nm (OD_{600}) reached 0.6. *L. reuteri* was grown on Mann-Rogosa Sharpe (MRS) plates containing 10 μg/ml kanamycin. For inoculation, *L. reuteri* was grown for 16 h in MRS broth. For viable counts of inocula and for the quantification of bacteria in mouse cecal contents, 10-fold dilutions were plated on SMAC agar or MRS agar plus 10 μg/ml kanamycin to enumerate EHEC or *L. reuteri* organisms, respectively. Media were from Difco (SMAC, MRS, and LB) or BBL (TSA).

Mouse experiments. Germfree Swiss-Webster mice, 3 to 4 weeks of age, were obtained from the breeding colony at the University of Michigan. These animals are maintained in soft-sided bubble isolators and are free of all bacterial, fungal, viral, and parasitic organisms. For experimental use, mice were aseptically transferred to sterile microisolator cages and housed in sterile laminar-flow hoods, where they can be maintained free from contaminating microorganisms for up to 4 weeks. Sterility was verified by terminal aerobic and anaerobic cultures and Gram stains of feces and cecal contents. All mice remained bacteriologically sterile except for the inoculated organism.

Mice were given sterile food, water, and bedding and were weighed daily. For EHEC infection, mice were orally inoculated once with 1.0×10^6 CFU of EHEC strain 86-24 in LB. For monocolonization with *L. reuteri*, mice were inoculated every other day with approximately 2.0×10^8 CFU/ml in MRS or potassium phosphate buffer (see below). For cocolonization with *L. reuteri* and EHEC, mice were inoculated with *L. reuteri* 1 day prior to EHEC inoculation and again either daily or every other day for the duration of the experiment.

To test the effects of reuterin, some trials were conducted with *L. reuteri* samples that were incubated with glycerol to induce reuterin production. For this, *L. reuteri* cells were collected by centrifugation, resuspended in 250 mM glycerol in 50 mM potassium phosphate, pH 7.4, and incubated at 37°C for 1 h prior to mouse inoculation. The production of ~100 mM reuterin was confirmed by bioassay (as previously described [27]). The treatment groups and number of mice in each group are shown in Table 1. Mice were weighed prior to the first inoculation and daily thereafter. They were euthanized 1 or 3 weeks after inoculation or when they became moribund. Mice that died or became moribund early were grouped according to their time of death. The 1-week group included mice that died or were euthanized from 6 through 8 days after inoculation, and the 3-week group included mice that were euthanized or died from 18 through 21 days after inoculation. Only seven EHEC-monocolonized mice survived to the 3-week time interval (Table 1).

Sample collection. At necropsy, mice were weighed and the cecum aseptically removed and weighed. Weighed aliquots of cecal contents were cultured for the quantification of bacterial counts, as described above. Samples of ileum, cecum, and kidney were immersion fixed in neutral-buffered formalin for histologic examination. Renal lesions were scored on a 0 to 3 scale according to severity (none, mild, moderate, or severe) and extent (none, focal, multifocal, or widespread) as previously described (9).

Statistics. Except as noted, groups were compared by Mann-Whitney U test (two groups) or by analysis of variance (ANOVA) with Bonferroni's correction for multiple groups. Categorical data were compared using the Fisher's exact test.

RESULTS

Mono- and coinoculation of mice by EHEC or *L. reuteri*. EHEC and/or *L. reuteri* were recovered from all mice inoculated with the respective strain regardless of inoculation protocol or experimental interval. No other aerobic or anaerobic bacteria were isolated from any mouse, and the uninoculated mice remained bacteriologically sterile. As previously reported (9), EHEC colonized mice well, and in monocolonized mice bacterial counts reached 10^{10} to 10^{11} CFU/g cecal contents by 1 week after inoculation (Fig. 1A). In mice monoinoculated with *L. reuteri*, bacterial counts 1 week after inoculation ranged from 6.95 $\log_{10}$ CFU/g cecal contents to 10.10 $\log_{10}$ CFU/g (mean, 8.51 ± 0.96) (Fig. 1) and did not differ significantly regardless of inoculation protocol (once [8.45 ± 1.17], every other day [8.20 ± 0.48], or daily [9.55 ± 0.29]) or experimental interval (1 or 2 weeks) (not shown).

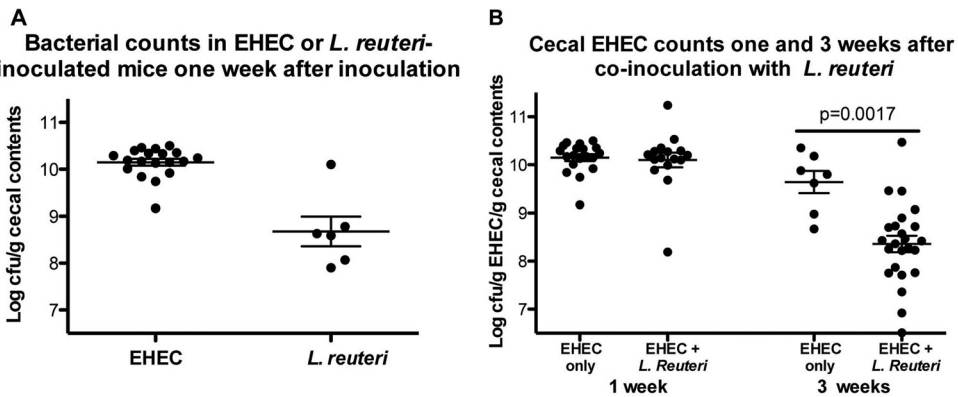

FIG. 1. Bacterial colonization density in cecal contents of mice inoculated with EHEC and/or *L. reuteri*. (A) Bacterial counts in EHEC- or *L. reuteri*-inoculated mice 1 week after inoculation. (B) EHEC counts in coinoculated mice 1 and 3 weeks after inoculation.

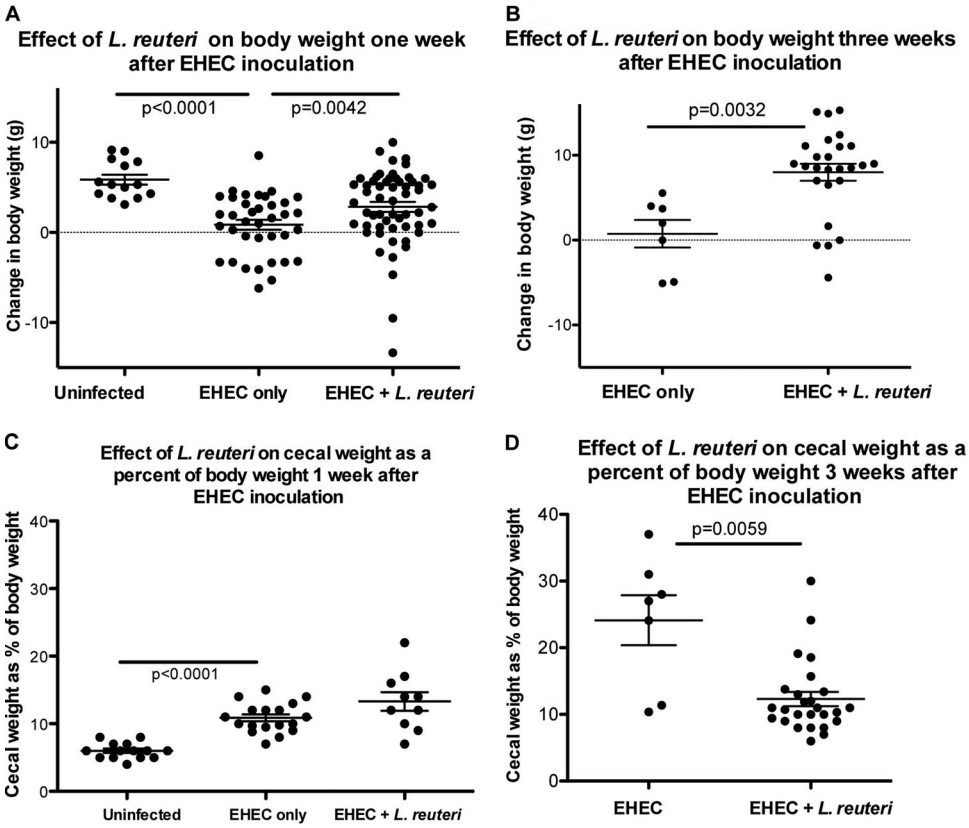

FIG. 2. Change in body weight (A, B) or cecal enlargement (C, D) in EHEC-infected mice with and without coinoculation with *L. reuteri* 1 (A, C) or 3 (B, D) weeks after EHEC inoculation.

Both bacterial species were recovered from all mice that were coinoculated with EHEC and *L. reuteri* regardless of the duration of the experiment. One week after inoculation, *L. reuteri* did not suppress colonization by EHEC (Fig. 1B), but by 3 weeks after inoculation, colonization by EHEC was significantly lower in *L. reuteri*-coinoculated mice than in the surviving monocolonized mice (Fig. 1B). The density of *L. reuteri* in coinoculated mice (8.10 ± 0.98 $\log_{10}$ CFU/g) did not differ from that of monoinoculated mice.

Suppression of weight loss in EHEC-infected mice by *L. reuteri* administration. Three- to 4-week-old mice are immature and normally grow at a rapid pace. Uninfected mice gained 2 to 10 g of body weight (mean, 5.9 ± 2.1 standard deviations [SD]) during the course of the first week of the experiment, depending on their weight at weaning (Fig. 2A). Similarly, *L. reuteri*-monocolonized mice gained between 0 and 10 g during the first week of infection (mean, 4.3 ± 2.8 SD, not significantly different from results for uninfected mice). None of these mice showed any clinical evidence of disease. In contrast, 31 of 32 EHEC-infected mice either lost weight or gained less than 5 g (mean, 0.8 ± 0.6) (Fig. 2A). The administration of *L. reuteri* partly ameliorated weight loss in EHEC-infected mice 1 week after inoculation (Fig. 2A). By 3 weeks after inoculation, none of the seven surviving EHEC-infected mice had regained their lost weight, but most of the mice that had received *L. reuteri* had recovered and gained between 6 and 15 g (Fig. 2B). This is similar to the expected weight gain of 10 to 12 g in germfree Swiss Webster mice between weaning and 6 weeks of age.

***L. reuteri* treatment suppresses cecal enlargement caused by EHEC infection.** In germfree mice, EHEC causes typhlitis characterized by cecal enlargement due to increased luminal fluid accumulation (9). In non-germfree mice, the cecum accounts for approximately 1% of the body weight, and in uninfected germfree mice, the cecum accounts for 5 to 10% of the body weight (9). At 1 week after inoculation, ceca of EHEC-infected mice were significantly larger than ceca of uninfected mice, regardless of *L. reuteri* cocolonization (Fig. 2C). By 3 weeks postinfection (p.i.), however, cecal weight in EHEC-monocolonized mice had increased to $24.12\% \pm 3.74\%$ of body weight ($P < 0.001$ compared to 1 week and uninfected groups). In contrast, most of the *L. reuteri*-cocolonized mice had significantly less cecal fluid accumulation than did surviving EHEC-monocolonized mice (Fig. 2D). Cecal size in EHEC- plus *L. reuteri*-cocolonized mice was similar to the expected percentage of $7.2\% \pm 0.7\%$ of body weight in age-matched uninfected germfree mice (9).

***L. reuteri* treatment suppresses renal tubular necrosis.** Renal tubular necrosis was present in most EHEC-infected mice by 1 week after inoculation regardless of *L. reuteri* cocolonization but was not detected in uninfected mice or in mice monocolonized with *L. reuteri*. In mice monocolonized with EHEC, lesions ranged from mild and multifocal (score of 1; Fig. 3A) to severe and widespread, involving most of the renal cortex

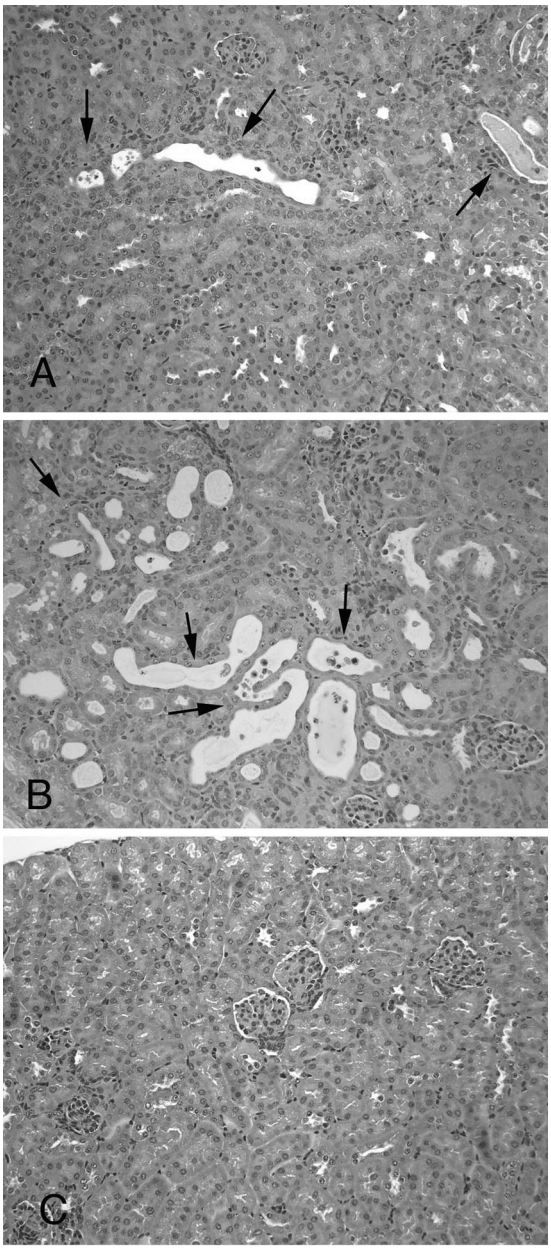

FIG. 3. Hematoxylin- and eosin-stained sections of kidney. (A) Section from an EHEC monocolonized mouse with mild acute tubular necrosis. Two tubules are mildly dilated and contain debris (arrows). (B) Section from an EHEC-monocolonized mouse with severe acute tubular necrosis. Most tubules in the field are dilated, and many contain cellular debris (arrows). (C) Section from an uninfected mouse. Renal tubules are normal. Original magnification, ×20.

(score of 3; Fig. 3B). Uninfected and *L. reuteri*-monocolonized mice had no renal lesions (Fig. 3C). In mice that survived to the 3-week time interval, lesions were significantly less severe in mice cocolonized with EHEC and *L. reuteri* than in mice given EHEC alone (Fig. 4). At that time interval, six of the seven surviving EHEC-infected mice had severe, widespread renal tubular necrosis, but only 8 of 26 mice treated with *L. reuteri* had severe lesions, and 4 mice had no evidence of tubular necrosis.

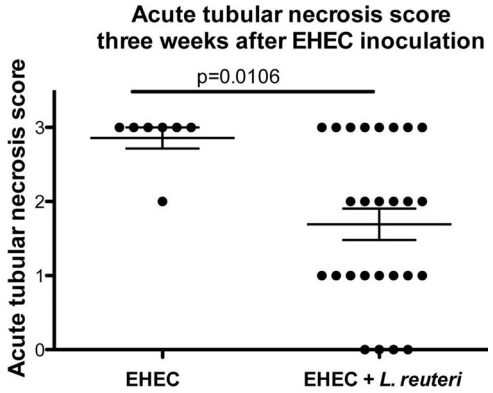

FIG. 4. Acute tubular necrosis scores of kidneys from mice infected with EHEC or coinoculated with EHEC and *L. reuteri* and examined 3 weeks after EHEC inoculation.

Recovery from disease in *L. reuteri*-treated mice. The evaluation of body weight in *L. reuteri*- and EHEC-cocolonized mice 3 weeks after inoculation revealed two groups of animals (Fig. 2B). Most of the mice appeared to have recovered and gained between 6 and 15 g during the 3-week course of the experiment, and they were of normal body weight for their age. In contrast, 5 of the 27 mice either lost weight or gained less than 1.7 g. This suggests that 22/27 mice responded to *L. reuteri* therapy and were recovering from EHEC-associated disease. Cecal size correlated well with body weight change (Fig. 5A), supporting this interpretation. The five mice that did not gain weight (the nonresponders) had significantly greater cecal fluid accumulation than the 22 mice that appeared to recover from disease and gained weight. Similarly, all five nonresponders had severe renal tubular necrosis (score of 3) compared to that of less severe lesions in all but three of the recovering mice (Fig. 5B).

Reuterin induction. Reuterin is an antimicrobial compound produced by *L. reuteri* that is effective against EHEC. To determine if reuterin alters the outcome of EHEC infection, mice were inoculated every other day with *L. reuteri* either alone or following incubation with glycerol to induce reuterin production (see Materials and Methods) and challenged with EHEC 1 day after the first *L. reuteri* inoculation. One (data not shown) or 3 (Fig. 6) weeks after inoculation, the groups did not differ in body weight, cecal weight, or acute tubular necrosis score, indicating that neither the presence of reuterin nor the inoculation medium used was responsible for the protective effect.

Twice-daily administration of *L. reuteri*. To determine if the increased frequency of the administration of *L. reuteri* enhances protection, a subset of mice were given *L. reuteri* twice daily beginning the day before EHEC administration and continuing for 7 days. In this group, reuterin was not induced prior to *L. reuteri* inoculation. In contrast to every-other-day administration, twice-daily administration of *L. reuteri* significantly prevented cecal enlargement 1 week after EHEC inoculation (Fig. 7A). Furthermore, of the five mice given *L. reuteri* twice daily, none developed renal tubular necrosis (Fig. 7B). However, body weight change (-0.92 ± 2.9) did not differ significantly from that of EHEC-monocolonized mice (0.85 ± 0.55). EHEC colonization in this group (10.4 ± 0.5 $\log_{10}$ CFU/g) did not differ from colonization in EHEC-monocolonized mice (10.2 ± 0.3 $\log_{10}$ CFU/g), indicating that protection from dis-

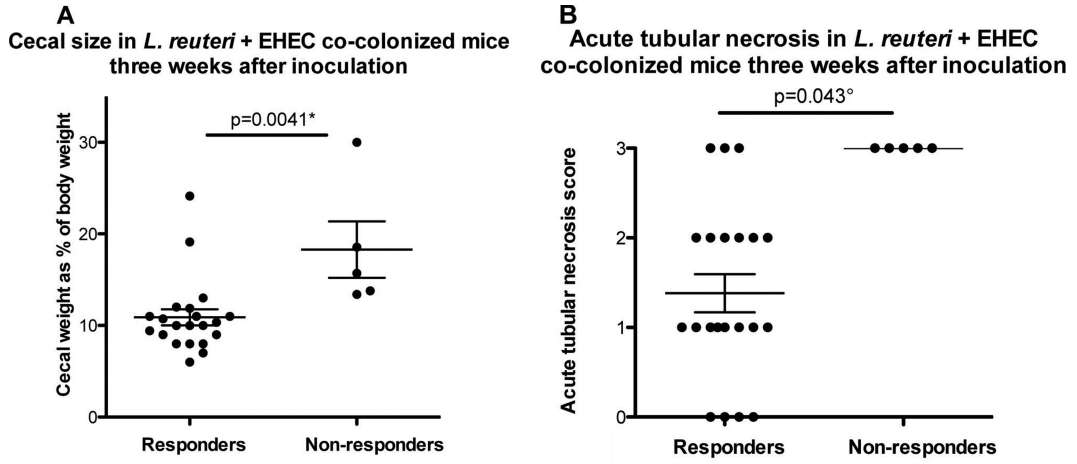

FIG. 5. Cecal size (A) and acute tubular necrosis score (B) in mice that were cocolonized with *L. reuteri* and EHEC and examined 3 weeks after colonization. Responders are cocolonized mice that had gained weight by 3 weeks after inoculation (see the text). Nonresponders are cocolonized mice that had failed to gain or had lost weight. *, Mann-Whitney U test; ○, Fisher's exact test.

ease was independent of the density of EHEC colonization (Fig. 7C).

DISCUSSION

The principal finding of this study is that *L. reuteri* administration suppresses both the colonization of EHEC and signs of disease due to EHEC in germfree mice. *L. reuteri* was effective regardless of inoculation protocol or reuterin induction, but there were some differences between treatment groups. Twice-daily inoculation with *L. reuteri* appeared to be the most effective of the protocols used. This group of mice was the only group that was completely protected from renal tubular necrosis due to EHEC, and these mice also were protected from cecal fluid accumulation by 1 week after inoculation. Because of the increased risk of contamination of gnotobiotic mice associated with multiple oral inoculations, twice-daily *L. reuteri* administration was not attempted for longer than 1 week. By 3 weeks after EHEC inoculation, most *L. reuteri*-treated mice were at least partly protected from disease as evaluated by body weight, cecal fluid accumulation, and renal tubular necrosis.

Interestingly, reuterin induction did not appear to affect the outcome of *L. reuteri* administration. Of the cocolonized mice treated every other day, there was no difference in the outcome in mice given *L. reuteri* with or without reuterin induction. Furthermore, at the 1-week sacrifice interval, twice-daily administration of *L. reuteri* protected mice even in the absence of the *in vitro* induction of reuterin. This indicates that *in vitro* induction prior to *L. reuteri* administration was not necessary for protection.

In this study, *L. reuteri* partly protected mice from both colonization by and disease due to EHEC. These results are similar to those of published studies demonstrating both colonization suppression and disease protection by other probiotic or nonpathogenic bacterial species, including certain strains of bifidobacteria (2, 10, 37), lactobacilli (23), clostridia (32), or commensal *E. coli* (11, 19). Colonization density by probiotic bacteria in those studies generally was similar to that of *L. reuteri* in the current study (2, 10, 11, 19, 32, 37), although reports ranged from 10^6 to 10^{10} CFU/g. The mechanisms whereby probiotics protect mice from disease due to EHEC are not well understood, although several possibilities have been investigated or proposed. The three main mechanisms cited for the ability of these organisms to suppress disease are colonization resistance against EHEC, the suppression of Shiga toxin production/activity, and the stimulation of host resistance. In most studies probiotic bacteria suppress EHEC colonization, hindering the definitive interpretation of the relative roles of bacterial density compared to those of other pathogenic mechanisms in the amelioration of disease. In the case of *L. reuteri*, however, we suggest that the suppression of EHEC colonization plays a minor role in protection, and that other mechanisms likely are more important. First, protection from disease manifestations was present in some groups either prior to or in the absence of the suppression of EHEC colonization. Mice given *L. reuteri* every other day were protected from weight loss by 1 week after inoculation, while the suppression of EHEC colonization was not evident until 3 weeks p.i. Similarly, mice given *L. reuteri* twice daily were protected from all manifestations of disease measured, but EHEC colonization was not affected. This interpretation also is supported by the finding that in our hands, a single inoculation of *Bacteroides thetaiotaomicron* colonized germfree mice at the same density as *L. reuteri* and slightly suppressed EHEC colonization, but it had no effect on disease due to EHEC (data not shown), suggesting that colonization suppression alone is not protective. Finally, in the mice in which EHEC colonization was suppressed, decreased EHEC colonization occurred only after recovery from clinical disease. By 3 weeks after inoculation, most of the *L. reuteri*-treated mice appeared to be recovering from disease, as indicated by the recovery of body weight and cecal function in most mice and the absence of renal tubular necrosis in some mice. It was only after recovery that decreased EHEC counts were detected. Thus, it is likely that in surviving mice the clearance of the pathogen was secondary to recovery from disease.

In addition to colonization suppression, several studies have suggested that probiotics also affect Shiga toxin production or activity. The major virulence factor for EHEC is the produc-

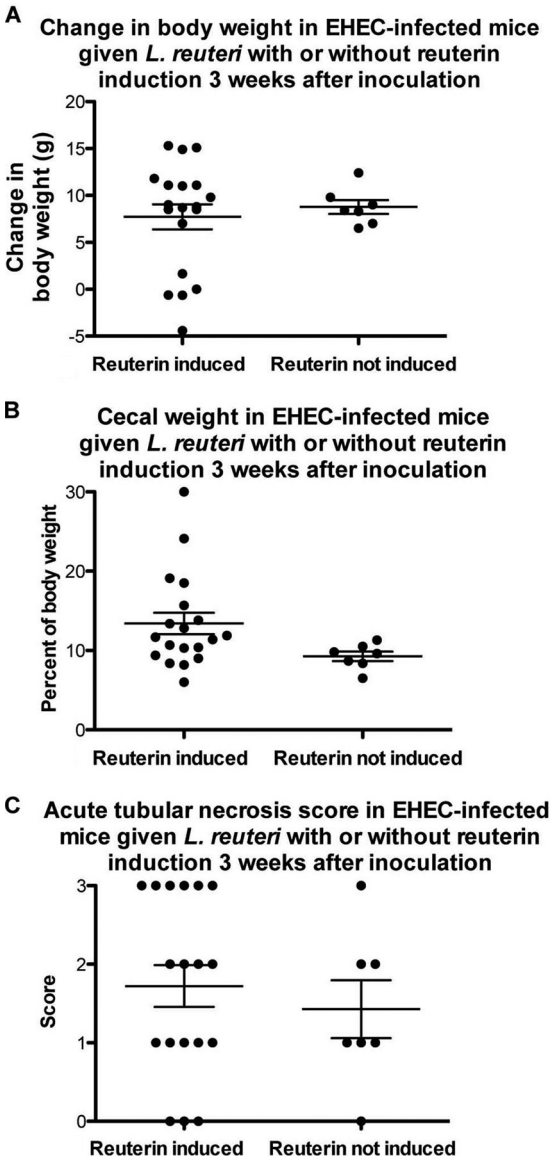

FIG. 6. Change in body weight (A), cecal weight (B), and acute tubular necrosis score (C) in mice cocolonized with EHEC and *L. reuteri* either with or without reuterin induction and examined 3 weeks after inoculation.

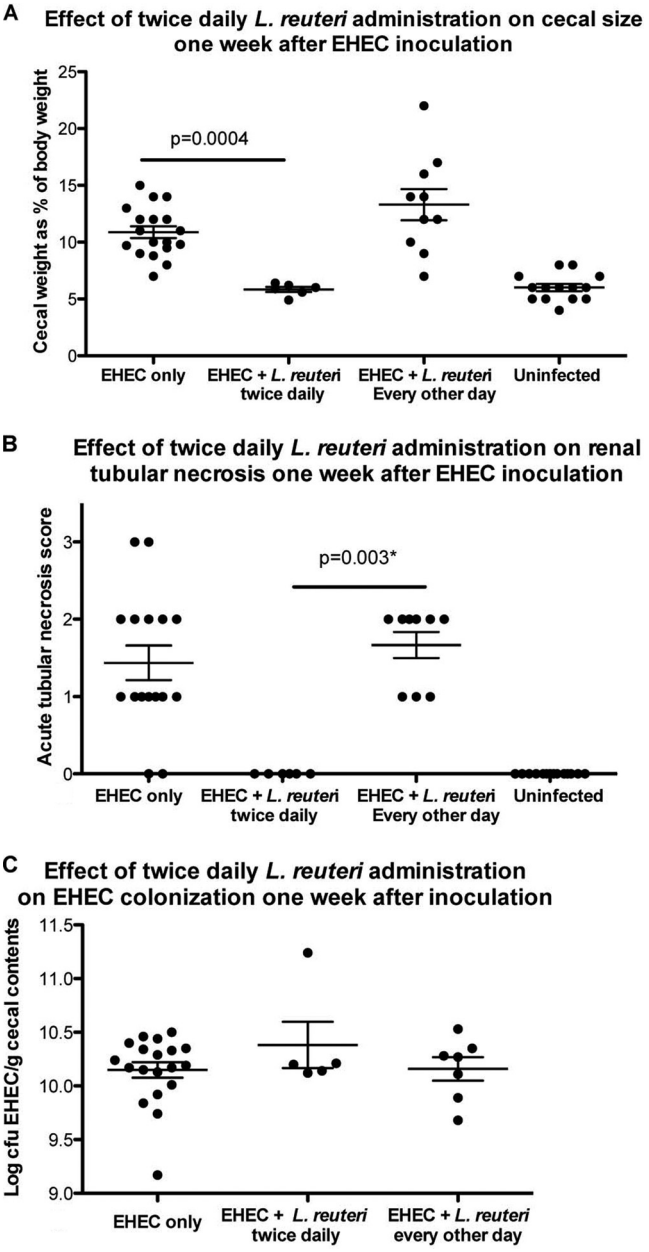

FIG. 7. Change in cecal weight (A), acute tubular necrosis score (B), and EHEC colonization (C) in EHEC-infected mice treated with *L. reuteri* either every other day or twice daily and examined 1 week after inoculation.

tion of Shiga toxins, most importantly Stx2 (3, 4). We have shown previously that weight loss, cecal fluid accumulation, and renal tubular necrosis due to EHEC in mice all are absolutely dependent on the production of Stx2 (9), and thus it is possible that *L. reuteri* treatment interferes with the production, release, or uptake of Stx2. Published evidence examining several different probiotic organisms supports this possibility. *In vitro* studies with probiotic organisms, including *L. reuteri* ATCC 55730, support a direct role for probiotics in suppressing Stx2 production (5). In addition, several studies noted above have shown that nonpathogenic *E. coli* (11), *Clostridium butyricum* (32), or bifidobacteria species (2) suppress Stx levels *in vivo*. The reduction in Stx2 levels in these models is somewhat difficult to assess, as colonization by EHEC also was affected, but a more-recent study showed that some strains of bifidobacteria can reduce disease and Stx2 production without showing a significant reduction in EHEC colonization (37). Finally, several studies have shown that at least *in vitro*, probiotics, including lactobacilli, may induce host protection mechanisms such as host inflammatory response, epithelial barrier function, or epithelial survival, thus conferring resistance to disease (16, 20). In a related model of proliferative colitis due to *Citrobacter rodentium* in young mice, protection from disease due to two *Lactobacillus* species was dependent on host T cells, suggesting that in this model disease resistance conferred by lactobacillus was host

mediated (12). Thus, mechanisms of protection vary among different probiotic bacterial species but probably include effects on both host and pathogen species.

The current study demonstrates that, like other probiotic species referred to above, *L. reuteri* ATCC PTA 6475 is effective in suppressing disease in an animal model of EHEC. Our results indicate that the increased frequency of administration increases protection, and that protection is independent of the density of *L. reuteri* in the intestine. Protection also appeared to be independent of EHEC colonization density, which was only minimally altered by *L. reuteri* administration, and then only late in the course of disease after recovery was under way. Reuterin induction in inocula did not enhance protection, but the determination of the role of reuterin induction *in vivo* will require the examination of *L. reuteri* mutants that are unable to synthesize reuterin. The role of *L. reuteri* in the alteration of EHEC toxin production and gene expression *in vivo* will require further investigation.

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