Probiotics Are Effective for the Prevention and Treatment of *Citrobacter rodentium*–Induced Colitis in Mice


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**Background.** Probiotics prevent disease induced by *Citrobacter rodentium*, a murine-specific enteric pathogen. Whether probiotics can be used to interrupt the infectious process following initiation of infection was determined.

**Methods.** C57BL/6 adult and neonatal mice were challenged with *C. rodentium*, and a probiotic mixture containing *Lactobacillus helveticus* and *Lactobacillus rhamnosus* was provided 1 week before bacterial challenge, concurrently with infection, or 3 days and 6 days after infection. Mice were sacrificed 10 days after infection, and disease severity was assessed by histological analysis and in vivo intestinal permeability assay. Inflammatory pathways and the composition of the fecal microbiome were assessed in adult mice.

**Results.** Preadministration and coadministration of probiotics ameliorated *C. rodentium*–induced barrier dysfunction, epithelial hyperplasia, and binding of the pathogen to host colonic cells in adults, with similar findings in neonatal mice. Upregulated tumor necrosis factor α and interferon γ transcripts were suppressed in the pretreated probiotic group, whereas interleukin 17 transcription was suppressed with probiotics given up to 3 days after infection. Probiotics promoted transcription of interleukin 10 and FOXP3, and increased follicular T-regulatory cells in pretreatment mice. *C. rodentium* infection resulted in an altered fecal microbiome, which was normalized with probiotic intervention.

**Conclusions.** This study provides evidence that probiotics can prevent illness and treat disease in an animal model of infectious colitis.

Probiotics are live microorganisms that, when consumed in sufficient amounts, confer a health benefit to the host [1]. Specific strains of probiotics successfully treat symptoms in patients with irritable bowel syndrome [2] and are effective in maintaining remission of ulcerative colitis [3]. In diarrheal disorders caused by an infectious pathogen, such as traveler’s diarrhea, or by an altered intestinal microbiome, such as antibiotic-associated diarrhea, probiotics can serve as an effective prevention strategy [4, 5]. In the developing world, where the burden of diarrheal disease is highest, probiotics reduce the fecal shedding of pathogens and decrease the duration of infectious diarrhea in children, making probiotics useful as a public health intervention [6].

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a gram-negative, noninvasive bacterium that is pathogenic in humans, causing acute hemorrhagic colitis [7] and persistent symptoms, including postinfectious irritable bowel syndrome [8]. Antibiotics are ineffective in treating EHEC infection and may promote the release of bacterial-derived Shiga toxins, thereby increasing the likelihood of hemolytic-uremic syndrome in at-risk populations [9]. Probiotics may serve as a potential option for managing EHEC infection.

As EHEC O157:H7 does not readily colonize mice, to test the potential use of probiotics in the prevention or treatment of bacterial-induced colitis, a model involving the murine-specific pathogen *Citrobacter*
rodentium was used [10]. Similar to the genome of EHEC O157:H7, the genome of C. rodentium contains the locus of enterocyte effacement pathogenicity island, which confers the ability to generate attaching and effacing lesions apically on colonocytes. Previously, we used the C. rodentium model to show that administration of Lactobacillus rhamnosus and Lactobacillus helveticus to mice prior to infection with C. rodentium reduces the severity of disease in both adult mice [11] and neonatal mice [12]. The majority of probiotic research has focused on the utility of probiotics as a preventive measure. In our previous studies, probiotics were given a week prior to infection and were then continued during the 10-day course of infection [11, 12]. The present study was undertaken to determine whether probiotics would ameliorate infectious colitis when given either concurrently with or following the infectious challenge.

MATERIALS AND METHODS

**Bacterial Strains**

A combination of probiotic bacteria comprising L. rhamnosus strain R0011 and L. helveticus strain R0052 (Lacidofil; Institut Rosell-Lallemand) was obtained as lyophilized powder and rehydrated in distilled water. C. rodentium strain DBS 100 (kindly provided by the late David Schauer, Massachusetts Institute of Technology) was grown on Luria-Bertani agar plates over-night at 37°C, followed by culturing in Luria-Bertani broth for 16 hours, yielding a final concentration of 10^9 colony-forming units (CFU) per milliliter.

**Mice and Protocols**

Female C57BL/6 mice (Charles River) aged 5–6 weeks were maintained in the containment unit of the Laboratory Animal Services facility at the Hospital for Sick Children (Toronto, Canada). As previously described [11], C. rodentium (10^8 CFU) was administered by orogastric gavage (100 µL). Luria-Bertani broth was administered as a sham control. Probiotics were provided ad libitum to mice at a concentration of 10^9 CFU/mL in drinking water [13]. This mode of delivery was selected to minimize stress associated with daily orogastric gavage, which can impact intestinal barrier function [14]. Probiotic solution was changed every other day to ensure a constant supply of viable organisms. For examination of neonatal mice, timed-pregnant female C57BL/6 mice (15–16 days; Charles River) were obtained, and previous methods were used for infection with C. rodentium and treatment of pups with probiotics [12].

Mice were divided into 6 groups: (1) sham infected, (2) C. rodentium infected, (3) C. rodentium infected plus probiotics initiated 1 week before infection, (4) C. rodentium infected plus probiotics initiated concurrently with infection, (5) C. rodentium infected plus probiotics initiated 3 days after infection, and (6) C. rodentium infected plus probiotics initiated 6 days after infection. Probiotics were continued in all groups until day 10 after infection. Additional adult mice were given probiotics for a week prior to infection, which was ceased either at the time of infection or 3 days after infection. All mice were sacrificed at 10 days after infection. All procedures and protocols were approved by the animal care committee at the Hospital for Sick Children.

**Quantitative Polymerase Chain Reaction Analysis of Proinflammatory and Anti-inflammatory Markers**

Full-thickness distal colon was homogenized and RNA harvested using a Trizol extraction protocol (Invitrogen). After treatment with DNase A (Invitrogen), samples were converted into complementary DNA, using an iSCRIPT complementary DNA synthesis kit (Bio-Rad; Mississauga, Canada). DNA was amplified by quantitative PCR (qPCR), using SsoFast EvaGreen Supermix and a CFX96 C1000 Thermal Cycler (Bio-Rad). Primers against mouse β-actin (housekeeping gene), interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 17α (IL-17α), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), forkhead box P3 (FOXP3), and transforming growth factor β (TGF-β) (Table 1) were used [15].

**Gut Microbiome Composition**

The colonic microbiome was analyzed using a 16S ribosomal RNA qPCR approach [15]. In brief, intracolonic fecal samples were collected at euthanization and frozen (−80°C). Bacterial DNA was extracted using a stool kit (Qiagen). DNA was amplified by qPCR, using SsoFast EvaGreen supermix and a CFX96 C1000 Thermal Cycler (Bio-Rad). Primer sets designed for 16S ribosomal RNA of bacterial species included those for Eubacteria (all bacteria; housekeeping gene), Bacilli, Bacteroides, Enterobacteriaceae, Firmicutes, Eubacterium rectale, and Lactobacillus/Lactococcus (Table 1). Colonization patterns were presented as percentage expression relative to total Eubacteria [15].

Bacterial populations in fecal samples were also evaluated using terminal restriction fragment length polymorphism (T-RFLP) analysis. PCR amplification was performed in triplicate with PfuUltra II fusion HS DNA polymerase (Stratagene), using primers targeting bacterial genes 8F and 1492R [16]. The 8F primer was labeled with 6′ carboxyfluorescein (Centre for Applied Genomics, Hospital for Sick Children). PCR products were pooled, verified on 2.0% agarose, and purified using a gel extraction kit (Qiagen). Purified DNA (200 ng) was digested with 10 U of MspI (New England Biolabs) for 30 minutes at 37°C. Digestion products then underwent capillary electrophoresis, using an ABI 3130 Genetic Analyzer. Raw electropherograms were analyzed for artefacts, as described previously [16]. Phylogeny analysis, Shannon-Weiner diversity, and evenness were calculated using previously described methods [17].
**Fluorescein Isothiocyanate–Dextran Permeability Assay**

Intestinal epithelial barrier function was measured in vivo, using a 4-kDa fluorescein isothiocyanate (FITC)–dextran probe (Sigma–Aldrich) in serum. Mice were fasted overnight, followed by orogastric gavage of FITC-dextran (100 μL; 88 mg/mL in sterile phosphate-buffered saline) [18]. After 4 hours, mice were briefly exposed to gaseous carbon dioxide and killed by cervical dislocation. Blood was obtained via cardiac puncture and placed on ice. Samples were centrifuged at 5000 rpm (4°C; 20 minutes), and serum was isolated. FITC-dextran concentrations were determined by fluorometry (PerkinElmer).

**Histology and Immunohistochemistry**

Distal colonic segments were cleaned of luminal contents, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Tissue was sectioned, stained with hematoxylin and eosin, and visualized using a Leica DMI 6000B microscope, Leica DFC420 camera, and Leica Application Suite Advanced Fluorescence software (Leica). Crypt depths were measured on coded sections. Final results represent the average of 10 crypt lengths per section of tissue from 2 nonadjacent colonic sections per animal.

Crypt cell proliferation, apoptosis, and T-regulatory cells (Tregs) were assessed by immunohistochemistry, using the following antibodies: anti-Ki67 (RM-9106; Thermo Fisher Scientific), anti-cleaved caspase-3 (AS175, 5A1E; New England Biolabs), and anti-FOXP3 (FJK-16S; Ebioscience). Relevant biotinylated secondary antibodies were used (Vector Laboratories) and visualized with a NovaRED system (Vector Laboratories). Ki67-positive and cleaved caspase-3–positive cells were quantified on coded sections by counting adherent, immunoreactive epithelial cells in 10 crypts in 2 nonadjacent sections of colon.

FOXP3-immunoreactive cells were counted in lymphatic follicles and normalized by the follicular area.

**Transmission Electron Microscopy**

Colons were obtained 6 days after infection and prepared for transmission electron microscopy (JEM-1011; JEOL USA), using previously described methods [19]. Images were acquired directly with a 1024 × 1024–pixel CCD camera system (AMT Corp).

**Statistical Analyses**

Results are expressed as mean value ± standard error of the mean. Comparisons were performed using 2-way analysis of variance and the unpaired Student t test, where indicated.

**RESULTS**

**Preventive and Concomitant Probiotic Administration Both Inhibit C. rodentium–Induced Colonic Epithelial Cell Hyperplasia and Barrier Dysfunction**

As previously established [11], compared with sham-infected mice (Figure 1A), infection with C. rodentium caused marked colonic epithelial hyperplasia (Figure 1B). Pretreatment and cotreatment with probiotics inhibited the hyperplastic response to C. rodentium infection (Figure 1C and 1D). By contrast, probiotic treatment starting at both 3 days (Figure 1E) and 6 days after infection (Figure 1F) were ineffective in preventing the hyperplastic response. Similarly, cessation of probiotic administration at the time of or 3 days after infectious challenge was ineffective (Figure 1G and 1H). These changes are quantified in Figure 1I.

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### Table 1. Primer Sequences Used in This Study

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**Kinetics of Probiotic Efficacy**

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Mechanisms by which epithelial cell hyperplasia is suppressed include increasing the cell apoptotic rate or decreasing colonocyte proliferation [10]. Colonic immunohistochemistry revealed no changes in cleaved caspase-3 immunoreactivity in any treatment groups (data not shown). By contrast, colonocyte proliferation was elevated, as indicated by an increase in Ki67 staining, in *C. rodentium*–infected mice, compared with sham-challenged controls (Figure 2A and 2B). Proliferation was inhibited with administration of probiotics both before and concurrently with bacterial challenge (Figure 2C and 2D). An antiproliferative effect was also visualized in the 3-day-postinfection probiotic group (Figure 2E) but not later. Quantification of Ki67 immunoreactivity is presented in Figure 2G.

*Citrobacter rodentium* infection resulted in an increase in intestinal permeability to a 4-kDa macromolecular fluorescent probe (Figure 2H) at the height of the colitis process [10]. As described previously [12], this increase in macromolecular permeability was inhibited with probiotic therapy initiated before *C. rodentium* infection. Concurrent administration of probiotics with the pathogen also maintained mucosal barrier integrity. By contrast, all other treatment regimens were ineffective at maintaining barrier function.

**Preventive and Concomitant Probiotics Prevent Mortality in *C. rodentium*–Infected Neonatal Mice**

*Citrobacter rodentium* infection results in severe, fatal colitis in neonatal mice, which can be ameliorated by probiotic pretreatment [12]. In the present study, *C. rodentium* infection resulted in severe weight loss and 100% mortality by day 10 after infection (Figure 3A and 3B). Pretreatment and cotreatment with probiotics were both effective at improving weight loss and mortality, whereas treatment 3 days after infection was ineffective (Figure 3A and 3B). In addition, *C. rodentium* infection causes marked barrier dysfunction in neonates. All 3 probiotic treatment regimens ameliorated barrier dysfunction in *C. rodentium*–infected neonatal mice at day 10 after infection (Figure 3D). By contrast, colonocyte hyperplasia responses in neonatal mice to *C. rodentium* infection were not impacted by the administration of probiotics (Figure 3C).

**Probiotics Diminish Proinflammatory Cytokines and Induce an Anti-inflammatory Profile**

To delineate a temporal relationship between probiotic administration and modulation of proinflammatory and anti-inflammatory factors, qPCR was used to detect the expression of helper T-cell 1 (TH1) cytokines (TNF-α and IFN-γ), a prototypical helper T-cell 2 (TH2) cytokine (IL-4), a helper T-cell 17 (TH17) cytokine (IL-17), a Treg marker (FOXP3), an

**Figure 1.** Probiotics are effective in preventing colonic epithelial cell hyperplasia when given before or concurrently with orogastric challenge with *Citrobacter rodentium*. Representative hematoxylin and eosin stained images of distal colonic sections taken 10 d after infection from the following groups: (A) sham infected, (B) *C. rodentium* infected, (C) *C. rodentium* infected plus probiotics initiated 1 wk before infection, (D) *C. rodentium* infected plus probiotics initiated concurrently with infection, (E) *C. rodentium* infected plus probiotics initiated 3 d after infection, (F) *C. rodentium* infected plus probiotics initiated 5 d after infection, (G) *C. rodentium* infected plus probiotics initiated 1 wk before infection and then stopped at infection, and (H) *C. rodentium* infected plus probiotics initiated 1 wk before infection and removed 3 d after infection.

**Figure 1 continued.** Quantification of mean crypt depth from each group of mice was performed (I). Scale bar = 220 μm. *P < .05 and **P < .01, by analysis of variance; n = 16 mice per group.
anti-inflammatory cytokine (IL-10), and a Treg-promoting factor (TGF-β; Figure 4A). Citrobacter rodentium–induced increases in gene expression of TNF-α and IFN-γ were prevented by pretreatment with probiotics but not by delivery of probiotics thereafter. Probiotics administered before, concurrently with, or 3 days after infection prevented a C. rodentium–induced increase in IL-17. As expected [20], TH2 inflammatory responses were not affected by C. rodentium infection.

Analysis of IL-10 transcript revealed an increase in gene expression over sham infection with probiotics given before, concurrently with, or 3 days after infection (Figure 4A). An increase in FOXP3 gene expression was observed in both the preventive and concurrent probiotic groups (Figure 4A). TGFβ expression was suppressed in C. rodentium–infected mice and was restored with the preventive probiotic regimen (Figure 4A). To determine physiological relevance, FOXP3+ Tregs were enumerated in lymphatic follicles, where a significant enrichment was observed only in the preventive probiotic regimen (Figure 4B).

**Preventive Probiotic Administration Reduces C. rodentium–Induced Alterations in the Intestinal Microbiome**

As probiotics mediate their positive effects partly through manipulating the composition of the enteric microbiome [15], their effect on C. rodentium–infection induced changes on the gut microbiome was assessed. As shown previously [15], C. rodentium infection resulted in an increase in Enterobacteriaceae, which was suppressed by probiotics provided prior to infectious challenge (Figure 5A) but not when administered concomitantly or following C. rodentium infection. C. rodentium infection was also associated with a significant decrease in Lactobacillus and Lactococcus species [21] that was prevented when probiotics were administered in advance of bacterial challenge (Figure 5B). Eubacterium rectale was also
elevated following *C. rodentium* infection, and this increase was suppressed with probiotic pretreatment (Figure 5C). No significant changes were observed in Bacilli, Firmicutes, or *Bacteroides* in any treatment group (Figure 5D–F). Segmented filamentous bacteria were not detectable in any of the samples tested (data not shown).

T-RFLP was used to assess the effects of probiotics on pathogen colonization and microbial diversity (Figure 5G). *Citrobacter rodentium* culture produced a fragment peak at 118 bp. The proportion of this peak (*p*) relative to the sum of all peak heights was highest in the infected group and in mice given probiotics starting at 3 and 6 days after infection. By contrast, *p* was reduced in mice receiving probiotics either prior to or concurrently with infection. In addition, following *C. rodentium* infection, T-RFLP profiles showed an overall decrease in microbial diversity, as indicated by a decrease in phylotype richness (number of distinct fragments), a lower Shannon-Weiner diversity score, and a decrease in evenness (a measure of dominance). These changes were restored to control levels with probiotic exposure, except in the 6-day-postinfection treatment group.

**DISCUSSION**

These findings are the first to demonstrate that probiotics are effective in treating infectious colitis when administered concurrently with or early during the course of an acute enteric infection. We have shown previously that oral administration of *L. rhamnosus* and *L. helveticus* as a prevention strategy decreases the severity of *C. rodentium*–induced colitis in both adult [11] and neonatal [12] mice. The findings herein describe physiological benefits of probiotic treatment given either concurrently with or up to 3 days following infectious challenge. This study, therefore, highlights the beneficial role of...
probiotics in interrupting enteric infectious disease progression, the caveat being the timing of both beginning and stopping the probiotic intervention have an impact on efficacy, similar to what has been described previously for the use of antibiotics to alleviate symptoms of *Campylobacter jejuni* infection in children [23].

Probiotics are an effective therapy against several human intestinal ailments, including acute infectious diarrhea, irritable bowel syndrome, and necrotizing enterocolitis in premature newborns [1]. Benefits of probiotic bacteria occur through various mechanisms. For example, *Lactobacillus reuteri* and *Lactobacillus plantarum* attenuate virulence by competitively excluding binding of pathogenic organisms to the host epithelium [24] and by acidifying the luminal environment [25]. Epithelial physiology can be modified by the presence of beneficial microorganisms, leading to enhanced innate immune

![Figure 4](https://academic.oup.com/jid/article-abstract/206/1/99/833328)
Figure 5. Probiotics and Citrobacter rodentium infection alter the composition of the fecal microbiome. The composition of the microbiome was evaluated using 2 complementary techniques: (1) quantitative polymerase chain reaction for specific 16S ribosomal RNA targets, expressed as a percentage of total
function, including the secretion mucins and defensins
[26, 27], and to enhanced intercellular tight junction integrity
[28]. Probiotics also alter adaptive inflammatory responses
to noxious stimuli and promote anti-inflammatory regulators
through activation of dendritic cells and Tregs [29] and by
elevating IL-10 secretion [30].

In this study, the efficacy of probiotics was assessed for
prevention, intervention, and treatment of murine infectious
colitis. The time points chosen correspond with key stages
in the pathogenesis of C. rodentium infection, including
initial infection, colonization of the colon (day 3 after
infection), and initiation of the inflammatory process (day 6
after infection) [31]. It is, therefore, not surprising that
probiotics had divergent effects on modulating different
aspects of the infectious process. For instance, probiotics
need to be provided early to prevent pathogen binding to
host enterocytes [24] and to modulate the adaptive immune
system [1]. By contrast, modulation of the innate immune
system and enhancement of intercellular tight junctions may
occur rapidly, thereby allowing for the beneficial benefits of
probiotics provided later during the course of enteric infection.

Colonic epithelial cell hyperplasia is a hallmark response of
colitis in mice to a variety of insults [32], including bacterial
infection [10]. The hyperplastic response to C. rodentium
is elicited through activation of host TH17 [33] and TH1 immune
responses. In adults, probiotics reduced colonocyte hyperplasia
when administered prior to or concurrently with C. rodentium
challenge. The hyperplastic mucosal response in the colon
to C. rodentium infection was mediated by an increase in
colonocyte division, in the absence of an altered apoptotic
response, as indicated by an increase in the number of
Ki67-immunoreactive epithelial cells [19, 34].

Both survival and body weight were significantly improved
by both pretreatment and cotreatment of probiotics in
C. rodentium–infected neonatal mice. In contrast to findings
in adult mice, colonic mucosal hyperplasia in response to
C. rodentium infection was not affected in neonatal mice pro-
vided probiotics.

*Figure 6.* Probiotics prevent binding of *Citrobacter rodentium* to host
epithelial cells when provided prior to or concurrently with infection.
Transmission electron microscopy photomicrographs of distal colon
demonstrating luminal bacteria in the following groups at 6 d after
infection: sham infected (A), *C. rodentium* infected (B), probiotics given
for 1 wk before *C. rodentium* infection (C), probiotics initiated at the
time of *C. rodentium* infection (D), and *C. rodentium* infection with
probiotics started 3 d after pathogen challenge (E). Open arrows indicate
*C. rodentium*, while solid arrowheads indicate lactobacilli. Scale bar = 2 μm
(n = 3 mice per treatment group).
anti-inflammatory pathways [38]. In the current study, the ability of probiotics to modulate T_{H}17 and T_{H}1 responses was demonstrated to be time dependent. Only a prevention strategy for probiotic administration was associated with suppression of IFN-γ and TNF-α transcripts 10 days after infection. T_{H}1 cell suppression is achieved by recruitment of Tregs by dendritic cells and secretion of IL-10 through an adaptive immune process [29, 39]. An induction of FOXP3 and IL-10 transcription, alongside an increase in FOXP3+ cells in colonic lymphoid follicles with probiotic pretreatment, supports the efficacy of probiotics in suppressing IFN-γ and TNF-α.

*Citrobacter rodentium* infection elicits a strong T_{H}17 response, characterized by an increase in the number of T_{H}17 cells and elevated IL-17 secretion [40]. With the exception of the 6-day-postinfection group, all other probiotic regimens reduced IL-17 transcripts. This finding indicates that probiotics suppress IL-17 gene expression yet allow for pathogen clearance, shown previously to be dependent on T_{H}17 cells [40]. Another recent study showed that the probiotic *Lactobacillus casei* suppresses IL-17 secretion without changing the number of T_{H}17 cells in antigen-stimulated mouse Peyer’s patch cell culture [41]. Therefore, probiotics may impact other IL-17-producing cell types, such as γδ T cells, natural killer cells, and granulocytes [42]. Future studies should identify which subsets of IL-17-secreting cells are affected by *C. rodentium* infection and probiotics.

The composition of the commensal enteric microbiome defends against bacterial pathogens [43]. Following *C. rodentium* infection, we [15] and others [21] have previously described the enrichment of Enterobacteriaceae and a decrease in lactobacilli/lactococci groups. In the current study, only pretreatment of probiotics suppressed the overgrowth of Enterobacteriaceae and supported control levels of lactobacilli/lactococci. Findings using T-RFLP demonstrated a global decrease in diversity following *C. rodentium* infection, which was modulated in all probiotic intervention groups, with the strongest protection provided with predadministration and coadministration of lactobacilli. Therefore, while specific changes in microbial communities may require early probiotic intervention, global changes in microbiome diversity can occur even with administration of probiotics later during the course of an enteric infection [44].

One mechanism by which probiotics mediate beneficial effects is by preventing the attachment of enteric pathogens to the intestinal epithelium [24]. The findings of the current studies show that probiotics provided either in advance or concurrently with an infectious challenge abrogate the binding of *C. rodentium* to colonocytes. These data were confirmed by using T-RFLP analysis, which showed that early intervention with probiotics suppressed the fecal load of *C. rodentium*. These data suggest that probiotics likely cannot displace intimately adherent pathogenic bacteria but can prevent initial pathogen attachment to mucosal surfaces.

In summary, the data described herein indicate that use of probiotics during the early stages of *C. rodentium* infection can protect against epithelial barrier dysfunction, mucosal hyperplasia, colonic inflammation, and pathogen colonization. Given the ability of probiotics to intervene against enteric infection in mice, these data support the need for additional patient-based research regarding the merits of employing probiotics in the setting of outbreaks of EHEC infection in humans.

### Notes

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**Potential conflicts of interest.** All authors: no reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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