

NOTES

Probiotics Reduce Enterohemorrhagic *Escherichia coli* O157:H7- and Enteropathogenic *E. coli* O127:H6-Induced Changes in Polarized T84 Epithelial Cell Monolayers by Reducing Bacterial Adhesion and Cytoskeletal Rearrangements

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The aim of this study was to determine if probiotics reduce epithelial injury following exposure to *Escherichia coli* O157:H7 and *E. coli* O127:H6. The pretreatment of intestinal (T84) cells with lactic acid-producing bacteria reduced the pathogen-induced drop in transepithelial electrical resistance. These findings demonstrate that probiotics prevent epithelial injury induced by attaching-effacing bacteria.

Escherichia coli serotype O157:H7 causes acute diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (18). The organism is the most common serotype identified in the group of enteric pathogens variously referred to as enterohemorrhagic *E. coli*, verotoxin-producing *E. coli*, and Shiga-like toxin-producing *E. coli* (15). Current therapy is limited to supportive treatment alone, because the use of antibiotics appears to increase the risk of systemic complications, such as acute renal failure occurring in hemolytic uremic syndrome, perhaps by promoting the release of toxin from the periplasm (34).

Enteropathogenic *E. coli* (EPEC) strains are non-toxin-producing bacteria associated with acute and protracted diarrhea in infants, particularly in the developing world. Both enterohemorrhagic *E. coli* and enteropathogenic *E. coli* bind to the surface epithelia, induce rearrangements of the cytoskeleton referred to as attaching and effacing lesions, and inject proteins (EspF) via a molecular syringe encoded by a type 3 secretion system that is targeted to intercellular tight junctions (16, 17).

Probiotics refer to a group of nonpathogenic organisms that are purported to have beneficial effects on health (26). A meta-analysis of randomized controlled trials provided evidence of the efficacy of lactic acid-producing bacteria for both the prevention and treatment of acute diarrhea in infants and young children (13). However, the precise mechanisms underlying these beneficial effects have not been clearly delineated. Therefore, the aims of the present study were to determine whether *Lactobacillus* species prevent injury to polarized intestinal T84 cell monolayers induced by infection with *E. coli* O157:H7 and *E. coli* O127:H6.

E. coli O157:H7 strain CL-56 was originally isolated from a stool sample obtained from a child with hemorrhagic colitis

and hemolytic uremic syndrome (29). For comparative purposes, enteropathogenic *E. coli* strain E2348/69 (serotype O127:H6) was also employed. *Lactobacillus* strains, including *Lactobacillus acidophilus* strain R0052 and *Lactobacillus rhamnosus* strain R0011, were provided by Institut Rosell-Lallemand Inc. (Montreal, Quebec, Canada).

E. coli strains were grown overnight at 37°C in static, nonaerated Penassay broth (Difco, Detroit, MI), spun at 3,000 rpm for 5 min, washed with sterile phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS to a final concentration of 5×10^9 bacteria/ml. *Lactobacillus* strains were either used as concentrated industrial proprietary preparations or grown overnight at 37°C in static, nonaerated de Man, Rogosa, and Sharpe (MRS) broth (Difco), spun at 3,000 rpm for 5 min, and then washed and suspended in sterile PBS to a final concentration of 5×10^9 bacteria/ml. These two methods of preparation were used to determine whether the growth conditions of the *Lactobacillus* strains altered their probiotic effects. Tyn-dallized probiotics were prepared from industrially grown lactobacilli by heat treatment for 1 h at 70°C on three consecutive days and by gamma irradiation (71.3 krad for 1 h).

To determine if the effects of probiotics were solely due to the lower pH and lactic acid production, lactic acid (100 mmol; Sigma-Aldrich, St. Louis, MO) was added to the upper chamber of a 6.5-mm (0.4 μ m pore size) 12-well Transwell (Corning Glass Works, Corning, NY), and *E. coli* strain CL-56 (O157:H7) or E2348/69 (O127:H6) (10^7 CFU in 1.0 ml of tissue culture medium) was added to the lower chamber and incubated overnight at 37°C in 5% CO₂. There was no inhibition of *E. coli* growth by lactate. The pH levels of T84 culture medium during the 24-h period of incubation with the probiotic strains were not reduced below 6.0 (data not shown).

Microbial interactions with host epithelial cells were assessed using HEP-2 and T84 epithelial cells, which were purchased from the American Type Culture Collection (Manas-

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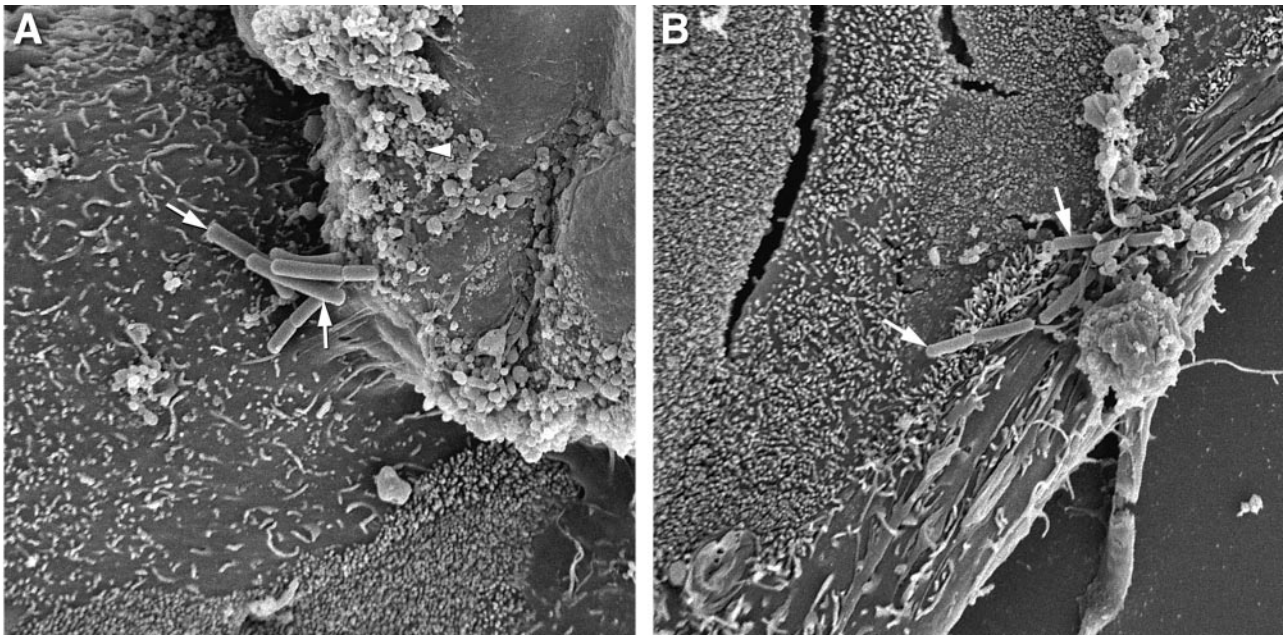


FIG. 1. Lactobacilli adhere to the surfaces of T84 epithelial cells. The scanning electron photomicrographs show *L. rhamnosus* strain R0011 (A) and *L. acidophilus* strain R0052 (B) grown in MRS broth adhering to cell surfaces (arrows). Approximate original magnification, $\times 4,300$.

sas, VA) and grown in tissue culture flasks according to established methods. Briefly, HEP-2 cells were cultured in minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum (Cansera International Inc., Rexdale, Ontario, Canada), 0.5% glutamine, 0.1% sodium bicarbonate, and 2% penicillin-streptomycin (all from Gibco). Cells were grown to confluence in 25-cm² flasks (Corning) or in LabTek chamber slides (Miles Scientific, Naperville, IL) at 37°C in 5% CO₂.

T84 cells were grown either in 25-cm² flasks (Corning) until confluence, in LabTek chamber slides (Miles Scientific), or in 6.5-mm 12-well Transwells (Corning) at 37°C in 5% CO₂ for 7 days. Cells grown in Transwells were cultured until the trans-epithelial electrical resistance (TER) reached a minimum of 1,000 Ω /cm². T84 cells were then preincubated for 6 h at 37°C with 10⁸ lactobacilli resuspended in 0.2 ml of tissue culture medium without antibiotics. Electrical resistance was measured with a Millicell probe (Millipore Corporation, Bedford, MA), and changes in response to exposure to bacteria were calculated as percentages of control values.

For infections of epithelial cells, the culture medium was replaced with antibiotic-free medium. Lactobacilli (10⁶, 10⁸, 10⁹, or 10¹⁰ bacteria) were then either coinfecting with *E. coli* (10⁷ bacteria) or preincubated with the host epithelium for 3 h or 6 h prior to the addition of the pathogenic bacterium. Infected cells were then incubated for up to 18 h at 37°C in 5% CO₂. The results are expressed as means \pm standard errors of the means. Analysis of variance (ANOVA) was employed to determine statistical differences between multiple groups.

Scanning electron microscopy was used to detect the attachment of *Lactobacillus* strains to the surfaces of T84 cells. T84 cells were treated with lactobacilli for 3 h at 37°C and then collected and prepared for electron microscopy as described previously (28). Briefly, cells were fixed overnight in parafor-

maldehyde and glutaraldehyde (4% and 1%, respectively, at pH 7.0), incubated in osmium tetroxide for 1 h at room temperature, and dehydrated in a graded series of ethanol (from 50% to 100%). Cells were then critically point dried and sputter coated with gold before visualization with a scanning electron microscope (model JSM 820; Joel Ltd., Boston, MA). Both *L. acidophilus* and *L. rhamnosus* adhered to the surfaces of both HEP-2 cells (data not shown) and T84 cells (Fig. 1).

Quantitation of the binding of *Lactobacillus* strains and *E. coli* to tissue culture cells was performed as described previously (31). Briefly, HEP-2 or T84 cells were grown overnight in antibiotic-free medium in 25-cm² flasks (Corning) or in LabTek chamber slides (Miles Scientific). Cells were either pretreated with probiotics for 3 h or 6 h prior to *E. coli* infection or coincubated with probiotics for 3 h at 37°C in 5% CO₂. Cells were then washed with sterile phosphate-buffered saline (Gibco) to remove nonadherent bacteria. Epithelial cells with adherent bacteria were trypsinized with 0.25% trypsin (Gibco) and centrifuged at 800 rpm for 5 min. The supernatant was discarded, and 1 ml of sterile distilled water plus 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) was added to lyse the eukaryotic cells (5). To selectively culture lactobacilli, Columbia blood agar plates (PML Microbiologicals, Mississauga, Ontario, Canada) were employed, whereas MacConkey agar (PML Microbiologicals) was used to detect the CFU of *E. coli* O157:H7 and E2348/69. Plates were incubated in the absence of CO₂ for 48 h at 37°C, and then individual colonies were enumerated. Staining with 10% Giemsa (Fisher Scientific, Pittsburgh, PA) was used as a complementary method to verify bacterial adherence to the tissue culture cells.

As shown in Fig. 2, pretreatment with *L. acidophilus* R0052 and *L. rhamnosus* R0011 (industrially rehydrated or grown in MRS broth) for 3 h resulted in a dose-dependent reduction in the adhesion of *E. coli* O157:H7 to HEP-2 (panel A) and T84

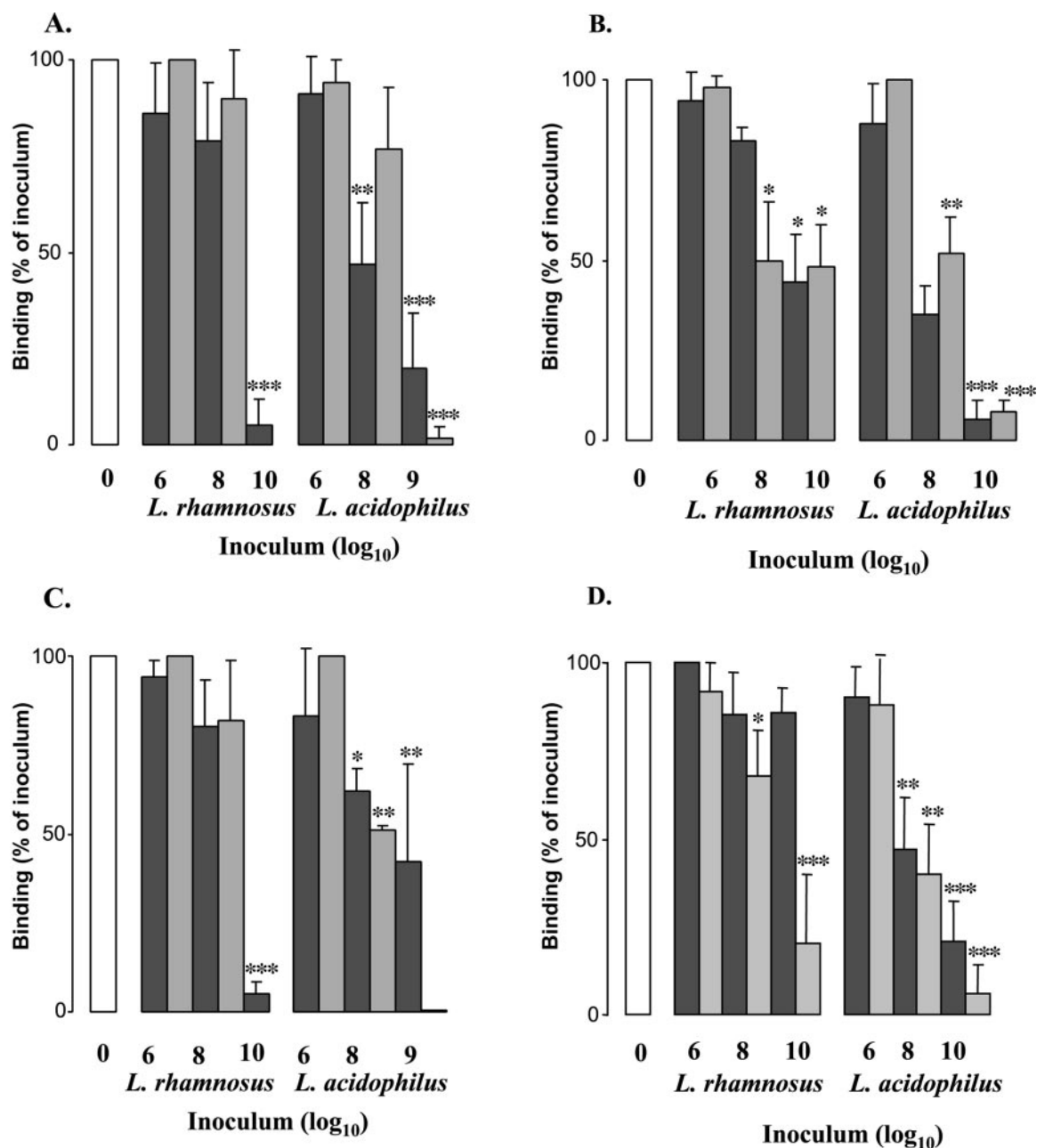


FIG. 2. Lactobacilli inhibit adhesion of *E. coli* O157:H7 strain CL-56 and EPEC strain E2348/69 (O127:H7) to epithelial cells. Quantitative adhesion assays showed that both *L. acidophilus* R0052 and *L. rhamnosus* R0011, grown either in MRS broth (dark gray bars) or using industrial methods (light gray bars), reduced the binding of *E. coli* O157:H7 and EPEC to the surfaces of HEp-2 cells (A and C, respectively) and to T84 epithelia (B and D, respectively) in a dose-dependent manner. Open bars represent the adhesion of enteric pathogens to epithelial cells in the absence of probiotics. *, $P < 0.05$ by ANOVA; **, $P < 0.01$ by ANOVA; ***, $P < 0.001$ by ANOVA (compared to control values).

cells (panel B). Similarly, a reduced adherence of EPEC strain E2348/69 was observed when host epithelial cells were pre-treated with lactobacilli prior to infection (Fig. 2C and D). The difference in the abilities of the *Lactobacillus* strains to prevent pathogenic adherence was both species and preparation dependent. In contrast to the case for preincubation with tissue culture cells, when lactic acid-producing bacteria were coincubated with the enteric pathogens there was no reduction in the adhesion of *E. coli* O157:H7 or EPEC strain E2348/69 to host epithelia (data not shown). In addition, neither preincubation

nor coincubation with Tyndallized probiotics prevented *E. coli* adherence to HEp-2 and T84 cells (data not shown).

To determine the mechanism of action of probiotics, supernatants from spent MRS broth were removed and filtered twice through a Millipore filter (0.2- μ m polysulfone membrane filter; Gelman Laboratories, Mississauga, Ontario, Canada). To ensure that viable organisms were not present, the medium was passed twice through a 0.2- μ m filter (Millipore). Plating onto blood agar plates was employed to ensure that viable bacteria were not present in culture supernatants.

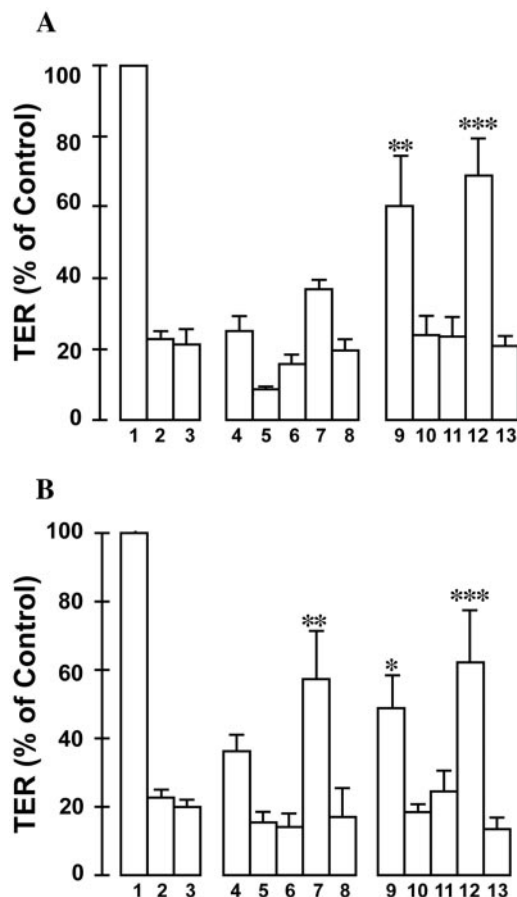


FIG. 3. Lactobacilli attenuate the drop in resistance induced by *E. coli* O157:H7 and EPEC infections. The pretreatment of T84 cells with *Lactobacillus* species (10^8 in 0.2 ml) for 3 h (A) or 6 h (B) prior to infection with *E. coli* O157:H7 or EPEC (10^7 in 0.2 ml) reduced the pathogen-induced drop in resistance. Lanes 1, uninfected T84 cells; lanes 2, cells infected with EPEC; lanes 3, Shiga-like toxin-producing *E. coli*-infected T84 cells; lanes 4 to 8, EPEC-infected cells preincubated with *L. rhamnosus* R0011 grown in MRS medium (lanes 4), *L. acidophilus* R0052 grown in MRS medium (lanes 5), *L. rhamnosus* R0011 grown under industrial conditions (lanes 6), *L. acidophilus* R0052 grown industrially (lanes 7), and Tyndallized *L. acidophilus* R0052 (lanes 8); lanes 9 to 13, *E. coli* O157:H7-infected T84 cells preincubated with *L. rhamnosus* R0011 grown in MRS medium (lanes 9), *L. acidophilus* R0052 grown in MRS medium (lanes 10), *L. rhamnosus* R0011 grown under industrial conditions (lanes 11), *L. acidophilus* R0052 grown industrially (lanes 12), and Tyndallized *L. acidophilus* R0052 (lanes 13). *, $P < 0.05$ by ANOVA; **, $P < 0.01$ by ANOVA; ***, $P < 0.001$ by ANOVA (compared to control values).

To prepare conditioned medium, T84 cells grown in T25 flasks were treated with either MRS broth-cultured or industrially rehydrated lactobacilli and then incubated at 37°C for 18 and 24 h. The conditioned medium was then removed, spun at 3,000 rpm for 5 min, and filtered twice through a Millipore filter (0.2- μ m polysulfone membrane filter; Gelman Laboratories).

As shown in previous reports (24, 25), the incubation of *E. coli* O157:H7 (10^8 CFU) or an equal amount of EPEC strain E2348/69 for 18 h induced an 80% reduction in the TER of T84 cells. In contrast, an equal number of lactobacilli, whether industrially rehydrated or grown in MRS broth, had no effect on the TER of polarized intestinal epithelia (data not shown).

The preincubation of viable probiotics for either 3 h (Fig. 3A) or 6 h (Fig. 3B) with T84 cells reduced the drop in TER induced by infection with *E. coli* O157:H7 or EPEC strain E2348/69. In contrast, the coincubation of *L. acidophilus* R0052 or *L. rhamnosus* R0011 with an equal number of pathogenic bacteria for 18 h did not prevent *E. coli*-induced reductions in the TER (data not shown). Viable lactic acid-producing bacteria were necessary to mediate the observed effects, since culture supernatants, conditioned medium, and Tyndallized preparations of *L. acidophilus* R0052 and *L. rhamnosus* R0011 did not prevent the drop in TER following infections of T84 cells with attaching-effacing *E. coli* enteropathogens.

To detect attaching-effacing lesions, indirect immunofluorescence using a murine monoclonal antibody against the F-actin bridging protein alpha-actinin was employed as described previously (14). Briefly, HEp-2 cells grown overnight on slides in 5% CO₂ at 37°C were washed with sterile PBS. Industrially rehydrated or MRS broth-cultured *L. acidophilus* or *L. rhamnosus* was added to a concentration of 10^8 CFU prior to or in conjunction with *E. coli* (10^7 CFU) infection. Cells were infected for 3 h and then fixed in 100% cold methanol (Caledon Laboratories, Georgetown, Ontario, Canada). A 1:100 dilution of anti-alpha-actinin (Sigma) was added to the cells and incubated at 37°C for 1 h prior to incubation with fluorescein isothiocyanate-conjugated AffiniPure goat anti-mouse alpha-chain-specific immunoglobulin M (Jackson ImmunoResearch Laboratories, Mississauga, Ontario, Canada). Cells were then mounted in slow-fade component A-glycerol-PBS (Molecular Probes, Eugene, OR), and slides were examined by alternating phase-contrast and immunofluorescence microscopy at a magnification of $\times 40$ (Leitz Dialuz 22; Leica Canada Inc., Willowdale, Ontario, Canada).

As shown in Fig. 4, indirect immunofluorescence microscopy demonstrated that viable probiotics reduced the number of foci of rearrangements of alpha-actinin, which was indicative of a reduced number of attaching and effacing lesions formed in response to *E. coli* O157:H7 and EPEC strain E2348/69 infections (14).

Using two strains of probiotic bacteria grown under two defined culture conditions, our findings indicate that the organisms did not adversely affect the functional integrity of intercellular tight junctions. In contrast to enterohemorrhagic *E. coli* serotype O157:H7 and enteropathogenic *E. coli* strain E2348/69, probiotics had no effect on the transepithelial electrical resistance of a polarized intestinal (T84 cell) monolayer. TER was employed as a marker of the integrity of intercellular tight junctions because it provides an electrical measurement that is inversely related to the permeability of the polarized epithelium to macromolecules such as mannitol and Cr-labeled EDTA (2). Moreover, preincubation, but not coincubation, of the monolayer with *L. acidophilus* and *L. rhamnosus* prevented the drop in TER induced by pathogenic *E. coli* infection. The findings of the present study are supported by previous observations reported using other probiotics, including nonpathogenic yeast. For example, *Saccharomyces boulardii* preserves the barrier function in T84 cells infected with either EPEC (7) or *E. coli* O157:H7 (8). However, in contrast to the results of the present study using lactic acid-producing bacteria, the results with *S. boulardii* did not correlate with a reduced binding of *E. coli* O157:H7 to T84 monolayers (8).

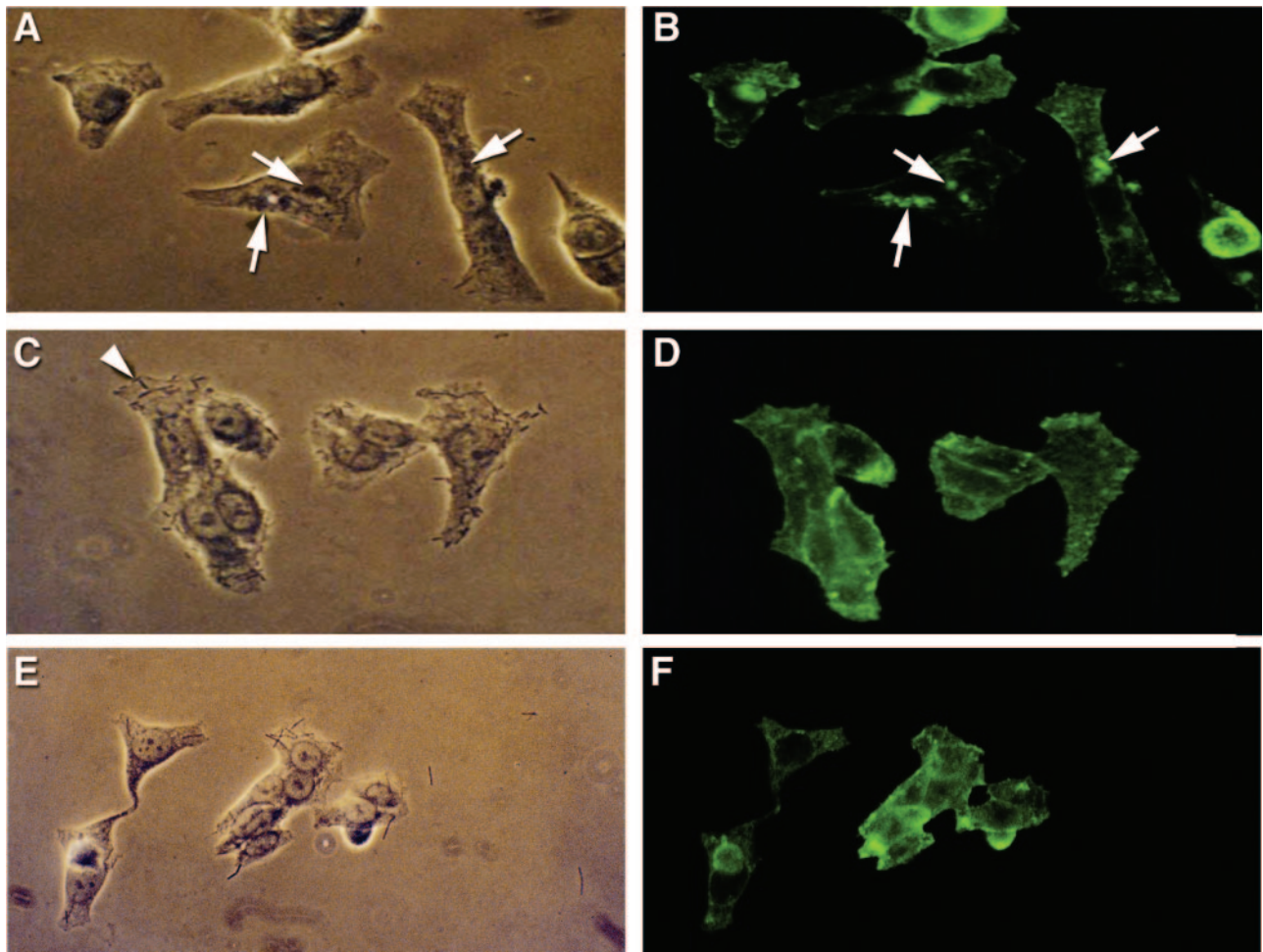


FIG. 4. Lactobacilli inhibit attaching and effacing *E. coli*-induced rearrangements of alpha-actinin in HEp-2 cells. Phase-contrast microscopy showed the binding of EPEC strain E2348/69 (arrows) to epithelial cells following infection for 3 h at 37°C (A). Corresponding fluorescence micrographs demonstrated the aggregation of foci of alpha-actinin underneath adherent EPEC strain E2348/69 (B). Phase-contrast microscopy demonstrated adherent *L. acidophilus* R0052 (C, arrowhead). Corresponding fluorescence microscopy (D) showed a negative alpha-actinin response. Phase-contrast microscopy showed reduced *E. coli* O157:H7 adhesion (E) and alpha-actinin reorganization (F) in HEp-2 cells coincubated with *L. acidophilus* R0052. Approximate original magnification, $\times 40$.

In the present study, viable probiotics provided a barrier which reduced the response of the host epithelium to pathogenic infections. Culture supernatants and Tyndallized bacteria did not provide a comparable beneficial effect. Previous studies support the need for viable probiotics. For example, Gotteland et al. (10) showed that heat-killed *Lactobacillus* GG does not mediate the protective effect on gastric damage induced by a nonsteroidal anti-inflammatory agent. Resta-Lernert and Barrett (27) reported that live bacteria are required for probiotics to protect intestinal epithelial cells in tissue culture from adverse effects induced by enteroinvasive *E. coli*.

The adhesion of lactobacilli to receptors on surface epithelial cells could compete for binding sites with enteric pathogens. It is also possible that lactic acid-producing bacteria reduce both the viability and the virulence properties of *E. coli* O157:H7 (6) and other diarrheagenic *E. coli* (19). In this study, we have demonstrated that these probiotics affect the virulence of *E. coli* O157:H7 and *E. coli* E2348/69 by factors other than their ability to reduce the pH or produce lactic acid. The ability of these probiotic strains to attenuate the pathogen-induced drop in TER

at neutral pH values strongly supports this contention. Several previous reports indicated that factors other than lactic acid produced by probiotics, including bacteriocins, proteinases, peroxides, and exopolysaccharides, could exert antibacterial effects (3, 32). However, nondigestible oligosaccharides have not proven to be effective alternatives to viable probiotics in experimental animal models of bacterium-induced diarrheal disease (22, 30).

Previous studies indicated that *Lactobacillus* species are able to adhere to the surfaces of intestinal epithelial cells in tissue culture (11). Previous work has shown, for example, that *E. coli* O157:H7 binds to the surfaces of *S. boulardii* cells (9). On the other hand, Hirano and coworkers (12) reported that *L. rhamnosus* blocks the internalization, but not the adherence, of *E. coli* O157:H7 in Caco-2 cells. Other lactic acid-producing bacteria, including *Lactobacillus gasseri*, *Lactobacillus casei*, and *Lactobacillus plantarum*, have no effect on either the binding or internalization of *E. coli* O157:H7 in Caco-2 cell monolayers (12). This study shows that *L. rhamnosus* and *L. acidophilus* have the ability to adhere to host epithelial cells and reduce the binding of both *E. coli* O157:H7 and *E. coli* E2348/69 to host epithelial cells.

Probiotics have also been employed with success in vivo. For example, in an infant rabbit model of *E. coli* O157:H7 infection, *L. casei* promotes immune responses against the *E. coli* cytotoxin and enhances the elimination of O157:H7 from the intestinal tract (23). Using a model of streptomycin-treated mice, Asahara et al. (1) showed that *Bifidobacterium breve* inhibits the consequences of *E. coli* O157:H7 infection in parallel with a drop in the luminal pH due to the production of high levels of acetic acid. Probiotics are also effective at reducing O157:H7 gut colonization in ruminants (36), which serve as the environmental reservoir for enterohemorrhagic *E. coli*. For humans, randomized trials have provided evidence of a beneficial effect of probiotics (20), including both the prevention and treatment of acute diarrhea in children (13, 33).

Boudeau et al. (4) reported that a probiotic reduces both the binding and internalization of adherent, invasive *E. coli* strains originally isolated from subjects with Crohn's disease. Similar to the findings reported in the present study, the preincubation of host cells with the probiotic was better than coinoculation at reducing binding of the *E. coli* strains (4). In summary, probiotic strains play an important role in attenuating host epithelial responses to pathogenic *E. coli* infections. Their role in modulating signal transduction responses in host epithelia infected with pathogenic bacteria, including enterohemorrhagic *E. coli* O157:H7 and *E. coli* O127:H6, is an important field warranting further investigation (21, 35).

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