Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression

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Mack, David R., Sonia Michail, Shu Wei, Laura McDougall, and Michael A. Hollingsworth. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am. J. Physiol.* 276 (*Gastrointest. Liver Physiol.* 39): G941–G950, 1999.—Probiotic agents, live microorganisms with beneficial effects for the host, may offer an alternative to conventional antimicrobials in the treatment and prevention of enteric infections. The probiotic agents *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG quantitatively inhibited the adherence of an attaching and effacing pathogenic *Escherichia coli* to HT-29 intestinal epithelial cells but did not inhibit adherence to nonintestinal HEp-2 cells. HT-29 cells were grown under conditions that induced high levels of either MUC2 or MUC3 mRNA, but HEp-2 cells expressed only minimal levels of MUC2 and no MUC3 mRNA. Media enriched for MUC2 and MUC3 mucin were added exogenously to binding assays and were shown to be capable of inhibiting enteropathogen adherence to HEp-2 cells. Incubation of *L. plantarum* 299v with HT-29 cells increased MUC2 and MUC3 mRNA expression levels. From these in vitro studies, we propose the hypothesis that the ability of probiotic agents to inhibit adherence of attaching and effacing organisms to intestinal epithelial cells is mediated through their ability to increase expression of MUC2 and MUC3 intestinal mucins.

MUC2; MUC3; *Escherichia coli* O157:H7; *Lactobacillus plantarum*; *Lactobacillus rhamnosus*

THE EPITHELIAL CELL layer of the intestinal tract is strategically located between the many microbes and antigens of the intestinal lumen and the inflammatory and immune effector cells of the host's lamina propria. The intestinal epithelial cell is capable of a regulated marked production of selected chemokines in response to invasive bacteria (39) and, as such, may be an important component in development of the host innate and acquired immune responses. Noninvasive enteropathogens, such as enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, also elicit increases in cytokines, such as interleukin-8, although not to the same magnitude as invasive pathogens (16). Another noninvasive enteric pathogen, enteropathogenic *E. coli* (EPEC), has been demonstrated to stimulate transepithelial migration of neutrophils (33) and cytokines such as interleukin-8 through the activation of the nuclear transcription factor (NF)-κB of infected cells (34). Furthermore, EPEC can produce factors that regulate the function of the intestinal mucosal immune system (19). Taken together, these findings suggest that there may be other protective responses provided by epithelial cells.

EHEC serotype O157:H7 and EPEC belong to a group of enteric pathogens that contain a pathogenicity island on the bacterial chromosome that induces cytoskeletal rearrangements in infected epithelial cells leading to the formation of a characteristic attachment and effacement lesion (28). Intestinal epithelial cell-derived mucins from animals bind to human EHEC and inhibit animal and human EPEC strain adherence in vitro (7). The binding of pathogens by mucosal epithelial cell mucins is an important defense mechanism for the host (7).

Mucins are high-molecular-weight glycoproteins synthesized and secreted by epithelial cells of a number of organ systems, including the intestinal tract. Mucins are characterized by their large size, high content of carbohydrates, and *O*-glycosidic bonds between *N*-acetylgalactosamine and either serine or threonine in the peptide backbone (7). Different mucin genes have been identified by isolation of partial cDNAs containing unique tandem repeat domains. Among the different human mucin genes, MUC2 and MUC3 are the predominant ileocolonic mucins. The MUC2 mucin polypeptide contains a repetitive peptide of 23 amino acids that is rich in threonine and proline residues, is heavily glycosylated, and is flanked by cysteine-rich domains (10). The MUC2 gene is expressed in goblet cells of the small and large intestine (3), and MUC2 mucins may be the major secreted mucin component of the colon (12, 38). In contrast, MUC3 has a 17-amino acid tandem repeat rich in threonine and serine residues with a cysteine-rich carboxy-terminal domain that shows homology to epidermal growth factor (11). MUC3 is not highly expressed in the colon (12, 38) but shows expression in both goblet cells and enterocytes of the small intestine (3).

Enteric pathogens possess a number of strategies to circumvent mucins that overlay epithelial cells (7). Mucin-pathogen interactions may be determined by the quantity and quality of epithelial cell mucins. Live, nonpathogenic bacteria that are fed to humans (i.e., probiotics) have been shown to prevent and/or improve intestinal infections with pathogens (5). Hypothesized mechanisms of colonization resistance by probiotic agents include direct actions against pathogens or their receptors and stimulation of the epithelial cell host

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acquired immune response (5). In this study, we sought to investigate interactions between probiotics and MUC2 and MUC3 intestinal mucins. The role of probiotic agents in inhibition of binding of attaching and effacing enteric pathogens with intestinal epithelial cells was investigated in an in vitro model.

MATERIALS AND METHODS

Bacteria and growth conditions. Stock cultures of EPEC strain E2348/69 (serotype O127:H6) and EHEC strain CL8 (serotype O157:H7) were maintained on trypticase soy agar slants (Becton-Dickenson Microbiology Systems, Cockeysville, MD) at 4°C. Stock cultures of *Lactobacillus plantarum* strain 299v and *Lactobacillus rhamnosus* strain GG (American Type Culture Collection 53103, Rockville, MD) were maintained on MRS agar (Difco Laboratories, Detroit, MI) at 4°C. Bacteria were kindly provided by Dr. James Kaper (E2348/69), Center for Vaccine Development (Baltimore, MD), Dr. Philip Sherman (CL8), The Hospital for Sick Children (Toronto, Canada), and Drs. Stig Bengmark and Bengt Jeppson (299v), Lund University (Lund, Sweden).

EPEC strain E2348/69 was originally isolated during an outbreak of infant diarrhea (20), and EHEC strain CL8 was isolated from stools of a child with hemorrhagic colitis and hemolytic uremic syndrome (4). These strains cause the attachment/effacement lesion and contain the *eaeA* gene (4, 14). EPEC and EHEC strains that cause the attachment/effacement lesion have a conserved genetic locus of enterocyte effacement (28).

L. rhamnosus strain GG was isolated in vitro from stool specimens of healthy humans (8). *L. plantarum* strain 299v came from sourdough and has phenotypic and genotypic similarity to a strain isolated in vivo from the human intestinal tract (15). Both *Lactobacillus* strains show the ability to colonize the human intestinal tract after oral administration (15, 35).

E. coli strains were grown overnight at 37°C in static, nonaerated Penassay broth (Difco), and *Lactobacilli* strains were grown overnight at 37°C in static, nonaerated MRS broth (Difco) to reach the mid-log growth phase. Bacteria were harvested by centrifugation at 2,500 *g* for 15 min at 20°C in a GPR centrifuge (Beckman Instruments, Palo Alto, CA). After two washes in sterile pH 7.4 Dulbecco's PBS (Life Technologies, Gaithersburg, MD) at 25°C, bacteria were resuspended in PBS. Quantification of bacterial suspensions was determined by using a standard curve (data not shown) of bacterial colony-forming units (CFU) on MacConkey agar (Difco) or MRS agar relative to visible absorbance (600 nm; Spectronic Genesys 5 spectrophotometer, Rochester, NY).

Cell growth conditions. HT-29 colonic adenocarcinoma cells (American Type Culture Collection) were grown in McCoy's 5a culture medium (Life Technologies). Some HT-29 cells were cultured under separate growth conditions to increase MUC3 mRNA expression and reduce MUC2 mRNA expression relative to HT-29 cells grown in original culture conditions (25). This was accomplished through a progressive transfer of some HT-29 cells from the regular McCoy's 5a culture medium to a glucose-free, 5 mM galactose-containing McCoy's 5a culture medium as previously described (25). HEp-2 human laryngeal epidermoid carcinoma cells (American Type Culture Collection) were grown in MEM (Life Technologies). Culture media for the various cell lines were supplemented with 10% heat-inactivated qualified fetal bovine serum (Life Technologies) and antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin sulfate, and 0.25 mg/ml amphotericin B; Life Technologies). The cultures were grown at 37°C in a humidified atmosphere with 5% CO_2 and were passaged after washing with Earle's balanced salt solution (Life Technologies) using either trypsin (HT-29 cells) or trypsin-EDTA (HEp-2 cells; Life Technologies).

Probiotic inhibition assays. Alteration in the adherence to epithelial cells of EPEC strain E2348/69 and EHEC strain CL8 by *L. plantarum* strain 299v was determined using modifications of an in vitro epithelial binding assay described previously (37).

For the in vitro adherence inhibition assay, 6×10^5 epithelial cells (HEp-2 or HT-29) were suspended in the appropriate antibiotic-free culture medium and transferred to individual wells of a 12-well polystyrene tray (Fisher). Cells were grown to confluence and washed three times with sterile, 37°C Hanks' balanced salt solution (Life Technologies) to remove culture medium and nonattached cells. Bacteria were added to 2.1 ml of the appropriate antibiotic-free cell growth medium. Different numbers of *L. plantarum* 299v in a 0.1-ml volume of PBS (pH 7.4, 25°C) were added 1 h before (preincubation studies) or at the same time (coincubation studies) as 10^5 CFU (in 0.1 ml of PBS, pH 7.4, 25°C) of a pathogenic *E. coli* strain of bacteria. After incubation for 3 h at 37°C, cells were washed four times with Dulbecco's PBS (pH 7.4, 37°C) to remove nonadherent bacteria. Cells with adherent bacteria were released from polystyrene wells using 0.1 ml of trypsin-EDTA (HEp-2 cells) or trypsin alone (HT-29 cells). After 10 min incubation at 37°C, ice-cold sterile PBS was added to each well, and the well contents were agitated to dissociate epithelial cells. Serial dilutions of adherent bacteria were plated on MacConkey agar and incubated overnight at 37°C for subsequent quantification by counting CFU. All experiments were run in triplicate.

Supernatants from the growth of *Lactobacilli* that were collected were filtered through a 0.2-μm filter (Gelman Sciences, Ann Arbor, MI) and then boiled for 10 min. Plating on MRS agar plates showed no evidence of bacterial growth following these procedures. In some experiments, these cell-free sterile supernatants from overnight growth of *L. plantarum* 299v were added in place of the *L. plantarum* 299v. Various dilutions of the supernatant in a total volume of 1 ml were added to HT-29 cells 1 h before the addition of 10^6/0.1 ml *E. coli* strain E2348/69. EPEC E2348/69 were added to 1.1 ml of the cell growth medium overlying the cells and were incubated for 3 h. Controls had 1 ml of MRS broth added.

In preliminary experiments, we evaluated the viability of *E. coli* coincubated with *L. plantarum* 299v. After harvesting of bacteria, EPEC strain E2348/69 were mixed with antibiotic-free McCoy's 5a culture medium and were added to 12-well plates without epithelial cells to reach a final concentration of 10^5/well. To one-half of the wells, 10^9/well *L. plantarum* 299v were added. After a 3-h incubation, quantification was made by CFU determinations after serial dilutions. No difference ($P = 0.37$) was observed between the number of *E. coli* CFU between those wells that were or were not coincubated with *L. plantarum* 299v. A crystal violet dye-binding assay (2) was also used in preliminary experiments to estimate the number of HT-29 cells after bacterial incubation. Identical cell numbers were quantified after a 3-h incubation of 1×10^5 E2348/69/well ($8.9 \times 10^5 \pm 2.9 \times 10^4$ cells/well) compared with coincubating 1×10^5 EPEC and 1×10^9 *L. plantarum* 299v ($1.0 \times 10^6 \pm 4.4 \times 10^4$ cells/well, $P = 0.1$) for 3 h. Culture media overlying HT-29 cells and HEp-2 cells contained phenol red to monitor pH. At the end of the incubation period with 10^9 *L. plantarum* 299v, the pH was turning acidic. The pH in the media overlying both HT-29 cells and HEp-2 cells were not different from each other.

Isolation of mucins. Cell culture supernatants overlying HT-29 cells grown in glucose culture medium to express high levels of MUC2 mRNA or HT-29 cells grown in galactose culture medium to express high levels of MUC3 mRNA (25) were processed by techniques previously described for purification of intestinal goblet cell mucin (24, 26). To minimize proteolytic degradation of the constituents in the culture media, 5 mM *N*-ethylmaleimide (Sigma-Aldrich), 2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 0.01% sodium azide (Sigma-Aldrich) were added along with 5 mM EDTA to the collected cell culture media. The culture media were centrifuged at 30,000 *g* for 30 min at 4°C to remove pelleted cellular and particulate debris. Components of the soluble supernatant were subdivided by buoyant density using isopycnic ultracentrifugation in cesium chloride (Fisher) with a starting density of 1.46 g/ml. The suspension was placed in polyallomer centrifuge tubes (DuPont, Wilmington, DE) and centrifuged in a Sorvall 50.2 Ti rotor (DuPont) at 105,000 *g* for 48 h at 4°C. After centrifugation, a needle was inserted to the bottom of the centrifuge tube, and eight fractions of equal volume were collected by using a peristaltic pump (Spectra/Chrom Macroflow pump; Spectrum Medical Industries, Los Angeles, CA). The eight fractions were analyzed with a refractometer (Abbe L; Milton Roy, Rochester, NY) to determine the buoyant density. Fractions were then placed in wetted cellulose dialysis tubing (50 kDa exclusion; Spectrum) and dialyzed against deionized water for 48 h at 4°C. Each fraction then underwent determination of nucleic acid content [optical density (OD) 260 nm], total protein by the method of Lowry with a prepared concentration of albumin (fraction V; Sigma-Aldrich) used as standard, and glycoprotein concentration using the periodic acid-Schiff assay with a prepared concentration of crude porcine mucin (Sigma-Aldrich) as the reference standard as previously described (24). Samples were partially lyophilized and were stored at −70°C until use in assays.

Mucin inhibition of bacterial adhesion assay. Examination of the inhibitory effects of cellular products secreted in their culture medium on binding of *E. coli* strain E2348/69 to HEp-2 cells was determined using modifications of the in vitro assay described (37). Briefly, the HEp-2 cells grown to confluence with antibiotic-free MEM in 12-well polystyrene trays (Fisher) were rinsed three times with sterile, 37°C Earle's balanced salt solution (Life Technologies) to remove culture medium and nonattached cells. Approximately 2.5×10^6 CFU *E. coli* E2348/69 in 0.1 ml of PBS (pH 7.4), 100 μg protein in a total volume of 0.1 ml of the different buoyant density fractions (dialyzed), and 2.1 ml antibiotic-free MEM were added to each well. After incubation for 3 h at 37°C, nonadherent bacteria were removed by six washes with PBS (pH 7.4, 37°C). Serial dilutions of adherent bacteria that had been dissociated from HEp-2 cells were plated for quantification by counting CFU. All experiments were run in triplicate.

Alteration of MUC2 and MUC3 mRNA expression by bacteria. HT-29 cells were grown to confluence (Falcon 3028; Becton-Dickinson, Franklin Lakes, NJ) in either the glucose-containing McCoy's 5a culture medium or glucose-free, galactose-containing culture medium (25). To ensure that the same relative number of bacteria was used in RNA harvest experiments as was used in binding studies, the number of bacteria added to flasks was based on cell culture site-contact surface area. That is, the surface contact area of flasks was calculated to be 45 times greater than the surface area of a well of 12-well plates. For studies using flasks, 45 times the number of bacteria were added than were added to each well of the 12-well plate to ensure there was not a greater concentration of bacteria per cell for the two systems. Therefore, 4.5×10^6 *E. coli* CFU/flask or 4.5×10^{10} *L. plantarum* 299v CFU/flask were added to culture flasks and incubated for 1 h before collection of total cellular RNA. In some experiments, 15 ml of a 1:25 dilution of sterile bacterial supernatant from *L. plantarum* 299v mixed with 15 ml of cell growth medium were added in place of the bacteria and were incubated for 1 h before HT-29 cellular RNA collection.

Total RNA preparation. Total cellular RNA was isolated from cells using a guanidine isothiocyanate-cesium chloride cushion ultracentrifugation technique (25). Briefly, cells were washed with Earle's balanced salt solution and were lysed with a solution containing 4 M guanidine isothiocyanate (Fisher Scientific, Fair Lawn, NJ), 50 mM sodium acetate (Sigma-Aldrich Chemical, St. Louis, MO), and 250 mM 2-mercaptoethanol (Fisher). Total RNA was recovered by centrifugation through 5.7 M cesium chloride (Amresco, Solon, OH) and 0.025 M sodium acetate cushion in a SW 41 Ti rotor (Beckman Instruments) at 32,000 rpm for 18 h at 20°C. The RNA pellets were suspended in 0.3 M sodium acetate and were precipitated with 2.5 vol of ice-cold ethanol.

Mucin and glyceraldehyde-3-phosphate dehydrogenase probes. The MUC2 probe (clone SMUC41) and the MUC3 probe (clone SIB 124; kindly provided by Drs. James Gum and Young Kim) cDNA inserts were cut from pBS-SK vectors with *Eco*R I (Life Technologies), gel purified, and labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Life Sciences, Arlington Heights, IL) using a random primer labeling kit (Amersham). Unincorporated nucleotides were removed by Sephadex-G50 (Pharmacia Biotech, Piscataway, NJ) filtration. Selected Northern blots were also hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe to ensure there was no degradation of mRNA samples. Specific activity was determined by scintillation counting. Probes contained a minimum of 5×10^8 counts $\cdot$ min^{-1} $\cdot$ μg DNA^{-1}.

Northern blot analysis. Twenty micrograms of total RNA of samples were subjected to electrophoresis on 1.2% agarose gels containing 0.66 M formaldehyde and ethidium bromide to allow for ultraviolet light visualization of 28S and 18S RNA. Transfer to nitrocellulose membrane was via capillary blotting. Probe hybridization of Northern blots was carried out at 42°C for 18 h as previously described (25). Signals corresponding to mucin and glyceraldehyde-3-phosphate dehydrogenase expression were detected by Phosphor screen autoradiography (Molecular Dynamics, Sunnyvale, CA). Screens were scanned by a PhosphorImager (Molecular Dynamics) and were quantified by area integration using ImageQuant software (version 3.3; Molecular Dynamics). Relative levels of 28S RNA were measured using a Computing Densitometer with ImageQuant software (version 3.3; Molecular Dynamics) from photographic negatives (type 665 film; Polaroid, Cambridge, MA) of agarose/formaldehyde gels taken under ultraviolet light.

Statistical analysis. Group data are expressed as means ± SE. Analyses between two groups were made by unpaired two-tailed *t*-tests, and analyses between multiple groups were determined using one-factor ANOVA with 95% confidence intervals. Post hoc ANOVA analyses were determined by Fisher's protected least-significant difference using the StatView version 4.0 software program (Abacus Concepts, Berkeley, CA).

RESULTS

Inhibition of adherence of E. coli by L. plantarum. Binding of 10^5 CFU/well *E. coli* E2348/69 to HT-29 cells was evaluated in the presence of increasing numbers of

L. plantarum 299v. As shown in Fig. 1, progressive inhibition of *E. coli* E2348/69 was noted when greater concentrations of *L. plantarum* 299v were added simultaneously to each well. In experiments in which the *L. plantarum* 299v was preincubated with the HT-29 cells for 1 h before the addition of E2348/69, a smaller inoculum of *L. plantarum* 299v was required to effect a significant reduction of E2348/69 epithelial cell adherence. That is, even 10^7 *L. plantarum* 299v/well ($8.5 \times 10^4 \pm 9.4 \times 10^3$ E2348/69 CFU/well) inhibited E2348/69 binding more than controls with no *L. plantarum* 299v/well ($1.2 \times 10^5 \pm 1.5 \times 10^4$ E2348/69 CFU/well, $P < 0.05$). However, when EPEC E2348/69 was added to HT-29 cells and incubated for 3 h before the addition of *L. plantarum* 299v for an additional 2-h incubation, no reduction in EPEC binding was demonstrated (data not shown).

Similar to our findings with the EPEC strain we used, adherence of EHEC strain CL8 to HT-29 cells was also decreased in the presence of increasing numbers of *L. plantarum* 299v. Binding of EHEC strain CL8 to HT-29 cells with 10^8 *L. plantarum* 299v/well ($1.0 \times 10^4 \pm 1.2 \times 10^3$ CL8 CFU/well) and 10^9 *L. plantarum* 299v/well ($2.9 \times 10^2 \pm 3.3 \times 10^1$ CL8 CFU/well) was less than with 10^7 *L. plantarum* 299v/well ($1.6 \times 10^4 \pm 1.9 \times 10^3$ CL8 CFU/well), 10^5 *L. plantarum* 299v/well ($1.6 \times 10^4 \pm 3.8 \times 10^3$ CL8 CFU/well), or no *L. plantarum*/well ($1.6 \times 10^4 \pm 1.9 \times 10^3$ CL8 CFU/well, $P < 0.05$).

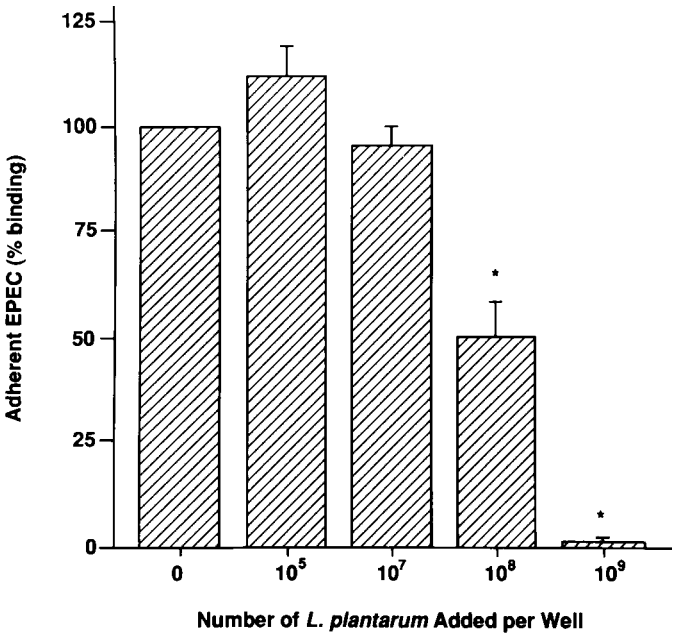

Fig. 1. Quantitative inhibition of enteropathogenic *Escherichia coli* (EPEC) adherence to HT-29 cells by *Lactobacillus plantarum* 299v. To each well of 12-well plates growing HT-29 cells, a 1×10^5 colony-forming unit (CFU)/well inoculum of EPEC E2348/69 was added with increasing amounts of *L. plantarum* 299v. After a 3-h incubation, EPEC adherent to HT-29 cells were quantified by CFU determinations as a percentage of adherent EPEC in wells on each plate that were without added *L. plantarum* 299v. Results are shown as means ± SE of 8 triplicate results (10^5 and 10^8, $n = 4$ experiments). *Compared with controls without the coincubated bacteria, there was decreased binding of EPEC with 10^8 and 10^9 *L. plantarum* 299v/well ($P < 0.05$).

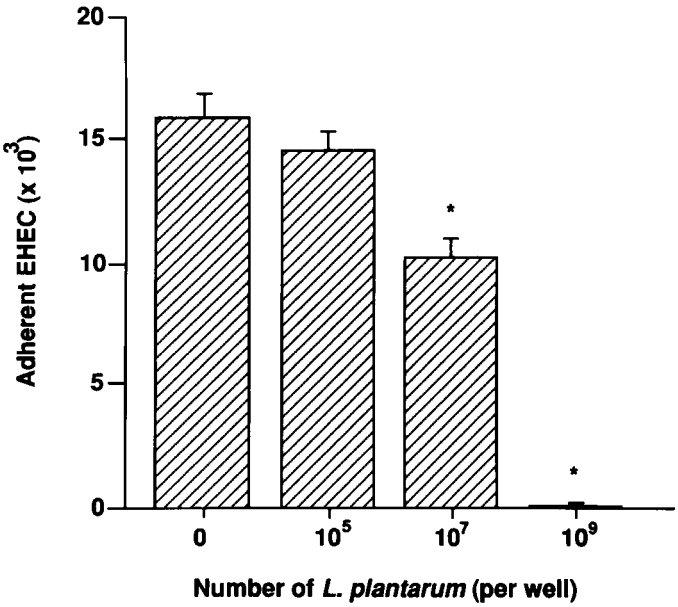

Fig. 2. Quantitative inhibition of enterohemorrhagic *E. coli* (EHEC) adherence to HT-29 cells by *L. plantarum* 299v. To each well of 12-well plates that contained HT-29 cells, a 1×10^5 CFU/well inoculum of EHEC O157:H7 was added 1 h after the addition of increasing amounts of *L. plantarum* 299v. Four hours after EHEC were added, EHEC adherent to HT-29 cells were quantified by determining CFU. Results are expressed as means ± SE of 12 triplicate results. *There was decreased binding of EHEC upon coincubation of 10^7 and 10^9 *L. plantarum* 299v/well ($P < 0.05$).

A 1-h preincubation with *L. plantarum* before the addition of 10^5 CL8 also decreased the required inocula of *L. plantarum* 299v to inhibit binding of EHEC strain CL8 to intestinal epithelial cells. Both 10^7 and 10^9 *L. plantarum* 299v/well inhibited binding compared with controls without *L. plantarum* 299v ($P < 0.05$) as shown in Fig. 2.

In those experiments in which the target epithelial cell for EPEC binding was HEp-2 cells, there was no quantitative inhibition with increasing amounts of *L. plantarum* 299v. As shown in Fig. 3, the yields of adhering EPEC after coincubation of 10^5 E2348/69/well with no *L. plantarum* 299v and 10^5, 10^7, and 10^9 *L. plantarum* 299v/well were similar ($P > 0.05$).

Dilutions of sterile supernatants collected after overnight growth of *L. plantarum* 299v were added to HT-29 cell culture media for 1 h followed by the addition of a 10^6 E2348/69 inoculum/well for a 3-h incubation. Adherent EPEC were then quantified as previously described. As shown in Fig. 4, undiluted supernatants and supernatants that were diluted 10-fold inhibited EPEC binding compared with supernatants diluted 100-fold and control wells without supernatant added ($P < 0.05$). Similar results were obtained using HT-29 cells grown in galactose-containing culture medium to increase MUC3 expression.

To determine whether *L. plantarum* 299v was unique in inhibiting adhesion of E2348/69 to HT-29 cells, another human probiotic agent, *L. rhamnosus* strain GG, was also used in some E2348/69 coincubation experiments. Both 10^9 *L. plantarum* 299v/well and 10^9 *L. rhamnosus* strain GG/well inhibited E2348/69 bind-

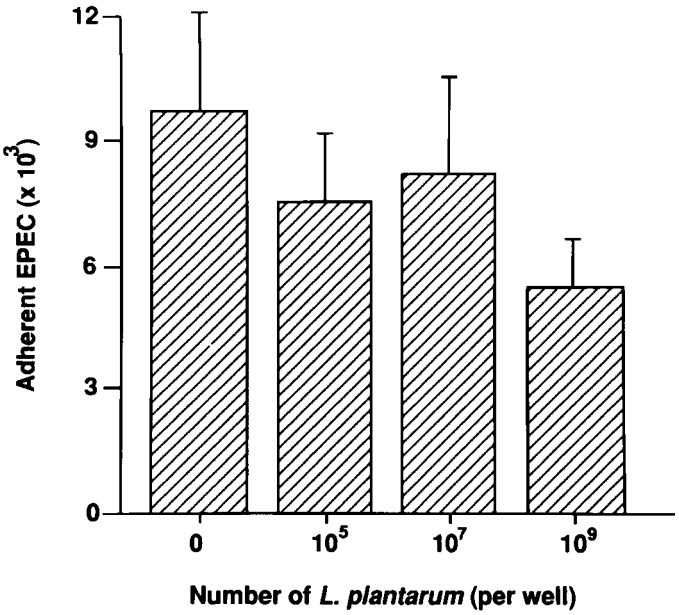

Fig. 3. Adherence of EPEC to nonintestinal epithelial cells during coincubation with *L. plantarum* 299v. To each well of HEp-2 cells, 1×10^5 CFU/well of EPEC E2348/69 was added with increasing amounts of *L. plantarum* 299v. After a 3-h incubation, EPEC adherent to HEp-2 cells were quantified by determining CFU. Results are expressed as means ± SE of 15 triplicate results. There were no differences in EPEC binding to these nonintestinal epithelial cells on coincubation with the indicated amounts of *L. plantarum* 299v ($P > 0.05$).

ing compared with control EPEC E2348/69 binding without *Lactobacillus* coincubation ($P < 0.05$) but were not different from each other in inhibiting EPEC binding to HT-29 cells ($P > 0.05$), as shown in Fig. 5.

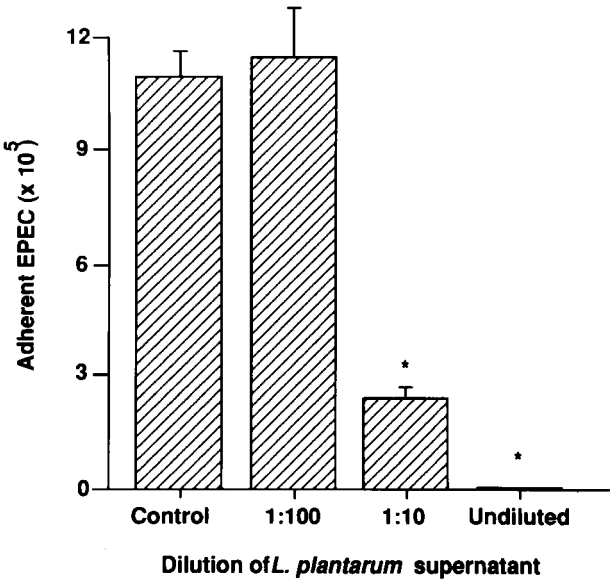

Fig. 4. Quantitative inhibition of EPEC adherence to HT-29 cells by sterile *L. plantarum* 299v supernatants. To each well of HT-29 cells that various dilutions of spent MRS broth of *L. plantarum* 299v culture had been added for 1 h was added 1×10^6 CFU/well of EPEC strain E2348/69. After a 3-h incubation of EPEC, bacteria adherent to HT-29 cells were quantified by CFU determinations. Results are means ± SE of 6 triplicate experiments. *There was decreased HT-29 cell EPEC binding for 1:10 dilution and undiluted supernatants compared with 1:100 dilution and controls without supernatant added ($P < 0.05$).

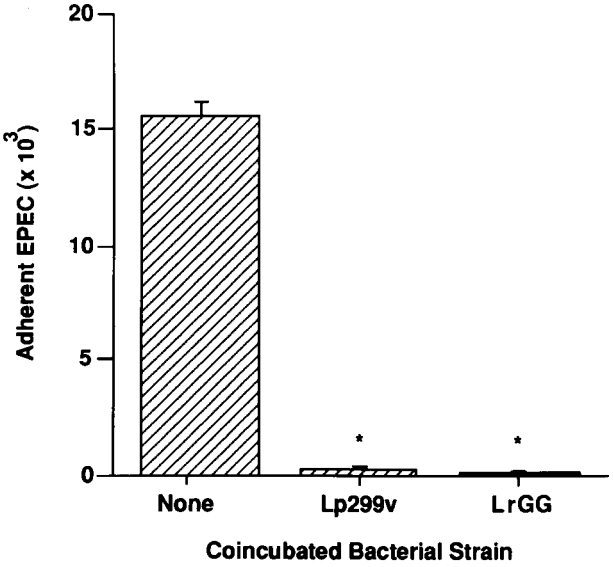

Fig. 5. Inhibition of EPEC adherence to HT-29 cells by probiotic agents. Each well of HT-29 cells received 1×10^5 CFU/well of EPEC E2348/69; some wells also received 1×10^9 of either *L. plantarum* 299v (Lp299v) or *Lactobacillus rhamnosus* GG (LrGG). After a 3-h incubation, EPEC adherent to cells were quantified by determining CFU. Results are expressed as means ± SE of 12 independent experiments. *Both Lp299v and LrGG decreased binding of EPEC compared with controls ($P < 0.05$); however, those reductions were similar to each other ($P > 0.05$).

MUC2 and MUC3 mRNA expression. Northern blots hybridized with MUC2 and MUC3 cDNA probes and 28S RNA levels of the blots are shown in Fig. 6. HT-29 cells grown in the glucose-containing culture medium showed high MUC2 and low MUC3 mRNA expression. The opposite pattern was seen in HT-29 cells grown in the glucose-free, galactose-containing culture medium in which a high MUC3 and low MUC2 mRNA expression was found. The MUC2 mRNA and MUC3 mRNA pattern detected by autoradiography is similar to that seen previously (25). HEp-2 cells expressed minimal levels of MUC2 mRNA, but MUC3 mRNA was not detected (Fig. 6). In separate experiments, there was no increased MUC2 mRNA or MUC3 mRNA expression detected in HEp-2 cells incubated with the equivalent number of 10^9/well of *L. plantarum* 299v (data not shown).

Inhibition assays with mucins. To examine whether MUC2 and MUC3 mucins added to the bacterial adhesion assay could inhibit EPEC adherence to HEp-2 cells, pooled supernatants from HT-29 cells were collected. Densities of the eight separated fractions of pooled supernatants from MUC2-enriched HT-29 cells grown in glucose culture medium ranged from 1.337 to 1.510 g/ml and from 1.338 to 1.506 g/ml for supernatant of MUC3-enriched HT-29 cells grown in glucose-free, galactose-containing culture medium. As previously for mucins from intestinal mucosa (24, 26), the most dense cesium chloride fraction (*fraction 1*) had a relatively high nucleic acid content (OD 260 nm > 0.45) compared with the middle-density fractions (OD 260 nm < 0.3). Comparable protein and glycoprotein profiles were obtained with corresponding buoyant density fractions, with progressively greater protein amounts found in

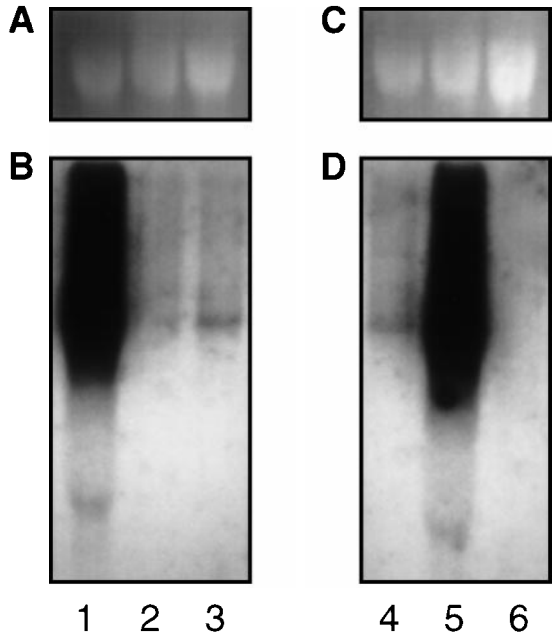

Fig. 6. Northern blot and analysis of MUC2 and MUC3 mRNA expression levels. Total RNA (20 μg/lane) from HT-29 cells grown in glucose-containing media (*lanes 1* and *4*), HT-29 cells grown in galactose-containing media (*lanes 2* and *5*), and HEp-2 cells (*lanes 3* and *6*) were separated by electrophoresis on 1.2% agarose/formaldehyde gels containing ethidium bromide. RNA was transferred to nitrocellulose membranes, and 28S RNA was visualized under ultraviolet light (*A* and *C*). Hybridization with a random-primed [^{32}P]cDNA probe of the MUC2 tandem repeat (*B*) or the MUC3 tandem repeat (*D*) was detected by autoradiography for 13 h at −70°C. HT-29 cells grown in glucose-containing media had high MUC2 mRNA expression, and HT-29 cells grown in glucose-free galactose-containing media had high MUC3 mRNA expression. HEp-2 cells showed minimal MUC2 mRNA expression and no MUC3 mRNA expression.

lighter buoyant density fractions. Glycoprotein content peaked in the middle fractions and was similar to previous characterizations of mucosal epithelial cell-derived mucins (24, 26, 37).

Quantitative inhibition of EPEC binding to HEp-2 cells was observed with material derived from buoyant density *fraction 4* of MUC3-enriched mucin. This fraction had a density and a glycoprotein-to-protein ratio consistent with mucin glycoproteins (24, 26). As shown in Fig. 7, quantitative inhibition of EPEC E2348/69 (2.5×10^6 bacteria/well) was demonstrated ($P < 0.05$).

To compare the adherence-inhibition properties of the buoyant density fractions from MUC2- or MUC3-enriched HT-29 cells, equal amounts (100 μg Lowry protein/well) of each fraction were incubated with 2.5×10^6 E2348/69/well. The evidence for enhanced expression of specific mucin proteins (increased mRNA expression levels) is necessarily indirect at this point because antibodies are not available that discriminate MUC2 and MUC3 production in spent culture media. We have previously found that, using these techniques, mucins are contained in the most dense fractions (26). This is a finding that would be compatible with the broad range of molecular weights that MUC2 and MUC3 mRNA have been found to have on agarose electrophoresis (10, 12, 13, 25). Similar to these previous findings, the least-dense fractions (*fractions 4–8*) derived from MUC2-enriched HT-29 cells did not inhibit EPEC binding; however, the more dense fractions (expected to contain mucins) inhibited EPEC binding (Fig. 8*A*). Similarly, the least-dense fractions (*fraction 7–8*) from supernatants of MUC3-enriched HT-29 cells that would not be expected to contain mucins (24, 26) did not inhibit the binding of EPEC to HEp-2 cells (Fig. 8*B*), but the more dense fractions did inhibit binding. Also, MUC3-enriched buoyant density fractions decreased E2348/69 binding to a greater extent than the corresponding MUC2-enriched fractions (Table 1).

Alterations in MUC2 and MUC3 mucin mRNA levels. Levels of MUC2 (Fig. 9*A*) and MUC3 (Fig. 9*B*) mRNA, normalized to 28S ribosomal RNA, were increased upon incubation of the HT-29 cells with an inoculum of probiotic bacteria that almost completely inhibited EPEC adherence. Compared with controls, relative expression levels of MUC2 mRNA for HT-29 cells grown in glucose culture medium incubated with 4.5×10^{10} *L. plantarum* 299v were increased (228 ± 48%, $P < 0.05$), but MUC2 expression was not altered upon coculture with 4.5×10^6 EPEC E2348/69 (146 ± 18%, $P > 0.05$; Fig. 9*A*). Similarly, for HT-29 cells grown in glucose-free galactose-containing culture medium (MUC3 induced), there was increased MUC3 expression for HT-29 cells incubated with *L. plantarum* 299v (207 ± 48%, $P < 0.05$) compared with controls, but expression by HT-29 cells incubated with EPEC E2348/69 (79 ± 24%, $P > 0.05$) was no different from controls (Fig. 9*B*).

Experiments ($n = 6$) using EHEC serotype O157:H7 yielded results similar to EPEC. Compared with con-

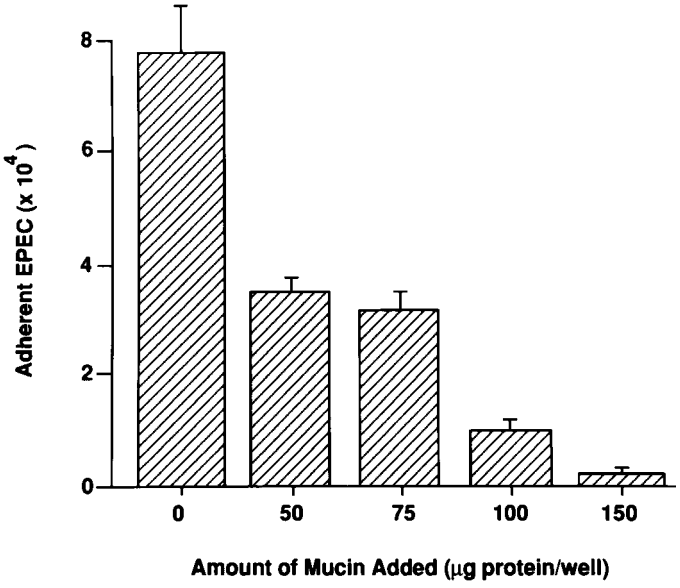

Fig. 7. Quantitative inhibition of EPEC adherence by mucin. To each well of HEp-2 cells, 2.5×10^6 CFU/well of EPEC E2348/69 were added with increasing amounts of material (protein) derived from MUC3-enriched buoyant density *fraction 4*. After a 3-h incubation, EPEC adherent to HEp-2 cells were quantified by determining CFU. Compared with PBS controls, all levels of protein employed in the assays diminished the capacity of EPEC to adhere to the HEp-2 cells ($P < 0.05$).

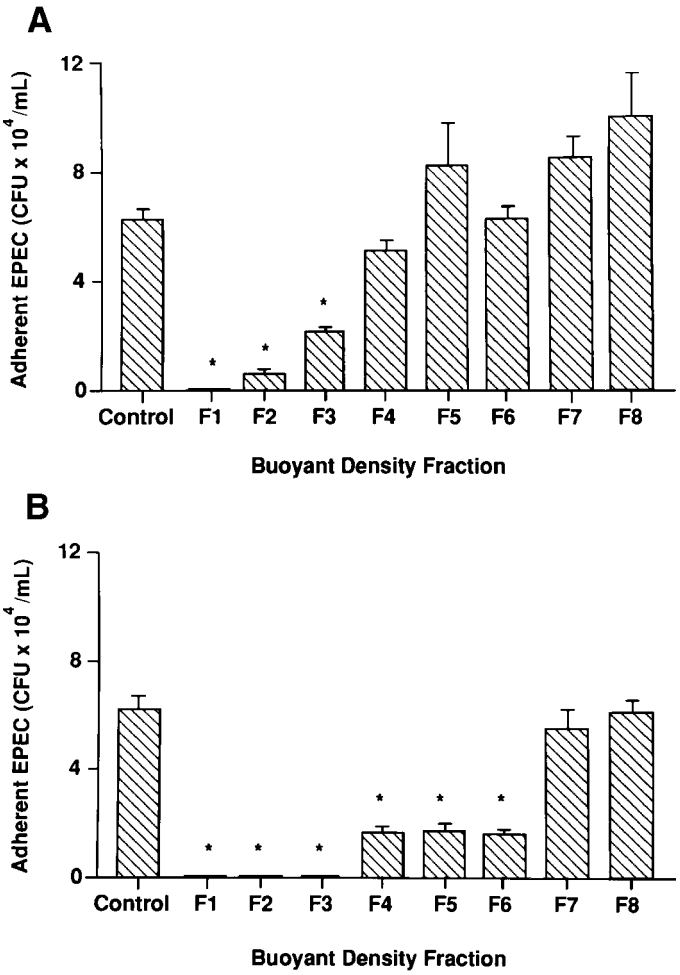

Fig. 8. Inhibition of EPEC adherence to HEp-2 cells by exogenous mucin. To each well of HEp-2 cells, 2.5×10^6 CFU/well of EPEC E2348/69 were added with an equal protein content (100 μg) from buoyant density fractions of MUC2-enriched spent HT-29 cell media (*A*) or MUC3-enriched HT-29 cell spent media (*B*). After a 3-h incubation, EPEC adherent to HEp-2 cells were quantified by determining CFU. Compared with PBS controls, binding of EPEC with high-density fractions derived from MUC2- and MUC3-enriched media (F1–F3) inhibited EPEC binding to HEp-2 cells ($P < 0.05$). In contrast, low-density fractions (F7 and F8) derived from both media did not inhibit EPEC binding compared with controls ($P > 0.05$). Middle-density fractions (F4–F6) derived from MUC3-enriched mucin fractions inhibited EPEC binding ($P < 0.05$), whereas middle-density fractions from MUC2-enriched fractions did not ($P > 0.05$). Results are shown as means ± SE of 4 triplicate experiments. *$P <$ 0.05 compared with control.

trols, relative expression levels of MUC2 mRNA from HT-29 cells grown in glucose culture medium (MUC2 induced) were greater for cells incubated with *L. plantarum* 299v (251 ± 49% of control, $P < 0.05$) but not with *E. coli* O157:H7 (187 ± 43% of control, $P > 0.05$). Similarly, MUC3 mRNA expression in HT-29 cells grown in galactose culture medium was increased with incubation of *L. plantarum* 299v (204 ± 50% of control, $P < 0.05$) but not with incubation of O157:H7 (116 ± 15% of control, $P > 0.05$) compared with controls.

In separate experiments, the addition of 4.5×10^6 EPEC strain E2348/69 did not lead to the detection of greater MUC2 mRNA levels above cells without bacteria added ($P > 0.05$). However, an inoculum of 4.5×10^{10} EPEC strain E2348/69 incubated for 1 h with HT-29 cells grown in a glucose medium showed increased MUC2 mRNA expression over both control cells and HT-29 cells with 4.5×10^6 EPEC ($P < 0.05$) added per flask as shown in Fig. 10. Similar findings with 4.5×10^{10} EPEC strain E2348/69 were also found with MUC3 expression in galactose-grown HT-29 cells. The addition of sterile supernatants from *L. plantarum* 299v also led to increased MUC2 expression (145 ± 10% of control, $P < 0.01$, $n = 6$) and MUC3 expression (149 ± 8% of control, $P < 0.01$, $n = 6$) in galactose-grown HT-29 cells (MUC3 enriched).

DISCUSSION

In the current study, two probiotic *Lactobacillus* strains were shown to inhibit in vitro adherence of EPEC or EHEC to the intestinal epithelial HT-29 cell line. Inhibition of EPEC binding to a nonintestinal epithelial cell line (HEp-2 cells), however, did not occur during coincubation studies with *L. plantarum* strain 299v. A number of factors are important for EPEC adherence to epithelial cells, including temperature, growth phase of EPEC, and pH conditions (17, 30). For these studies, standardized conditions for bacterial induction of the attachment and effacement lesion in epithelial cells included a constant number of EPEC added to each well, constant temperatures (37°C), and sodium bicarbonate buffering capacity in the cell growth medium. During the 3-h incubation, there was a change in the pH conditions with the greater inocula of the *L. plantarum* 299v as evidenced by changes in the phenol red indicator in the cell growth media. However, the observed change in pH was the same for studies with HT-29 cells and HEp-2 cells. The pH at the end of the incubation period is within the range of pH that allows for EPEC secretion of proteins necessary for the induction of the attachment and effacement lesions (17).

There are a number of possible explanations whereby probiotics effect benefit to the host. In this study, our experimentation showed that a direct antimicrobial effect of *L. plantarum* strain 299v against EPEC is unlikely. The inhibition of EPEC binding to the intestinal cell line (HT-29 cells) and lack of inhibition of binding to a nonintestinal epithelial cell target (HEp-2

Table 1. *Inhibition of EPEC binding by buoyant density fractions of MUC2- and MUC3-enriched media*

Buoyant Density Fraction	EPEC E2348/69 Adherence to HEp-2 Cells, ×10³ CFU/ml	
	MUC2-enriched media	MUC3-enriched media
2	6.6 ± 1.7	0.008 ± 0.002*
3	22.0 ± 1.3	0.410 ± 0.012*
4	52.0 ± 3.0	17.0 ± 2.2*
5	83.0 ± 16.0	18.0 ± 1.9*
6	63.0 ± 4.7	16.0 ± 1.1*

Values are means ± SE. EPEC, enteropathogenic *Escherichia coli;* CFU, colony-forming units. Equal amounts of protein (100 μg/well) of each buoyant density fraction were incubated with 2.5×10^6 E2348/69 bacteria/well in wells growing HEp-2 cells (see MATERIALS AND METHODS). *$P < 0.01$.

cells) disproves this possibility. Furthermore, direct coincubation of the two bacterial strains did not yield diminished numbers of viable EPEC numbers. Because EPEC strain E2348/69 can transfer its own receptor for intimate adherence to mammalian cells (18), interference by *Lactobacillus* strains with the epithelial cell

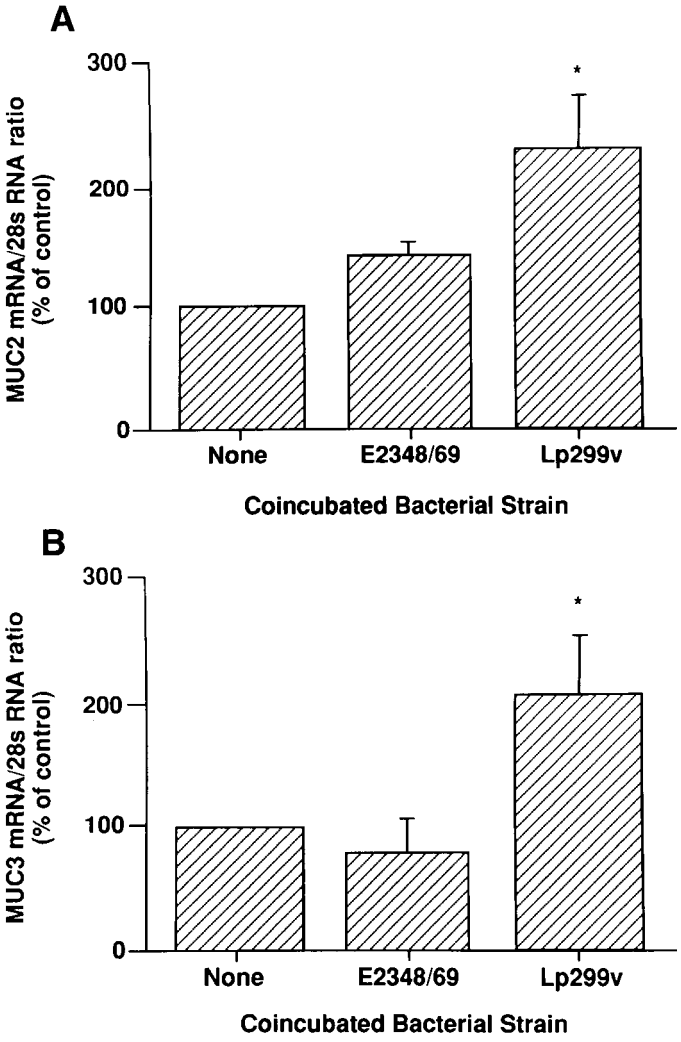

Fig. 9. Relative MUC2 and MUC3 mRNA expression levels after bacterial incubation. HT-29 cells were grown in glucose-containing media (*A*) or glucose-free, galactose-containing media (*B*). Bacteria were added to flasks in equivalent numbers to bacteria added to wells of the 12-well plates, based on a surface area of 10^5 EPEC E2348/69 or 10^9 *L. plantarum*. After a 1-h incubation, total RNA was collected and separated by electrophoresis on a 1.2% agarose/formaldehyde gel before being transferred to a nitrocellulose membrane. Hybridization using random-primed ^{32}P-labeled MUC2 and MUC3 cDNA probes of the respective tandem repeats was performed. Relative mucin mRNA levels were quantified from area integration of Phosphor screen autoradiography, normalized by dividing the densitometer values of photographic negatives for corresponding 28S RNA levels on agarose gels. MUC2 expression of HT-29 cells grown in glucose-containing media was increased in the presence of *L. plantarum* 299v ($P < 0.05$) but not with EPEC E2348/69 ($P > 0.05$) compared with controls with PBS alone (*A*). Similarly, MUC3 expression of HT-29 cells grown in glucose-free, galactose-containing media was increased in the presence of *L. plantarum* 299v ($P < 0.05$) but not with EPEC E2348/69 ($P > 0.05$) compared with controls with PBS alone (*B*). Results are shown as means ± SE from 5 separate experiments. *$P < 0.05$ vs. control.

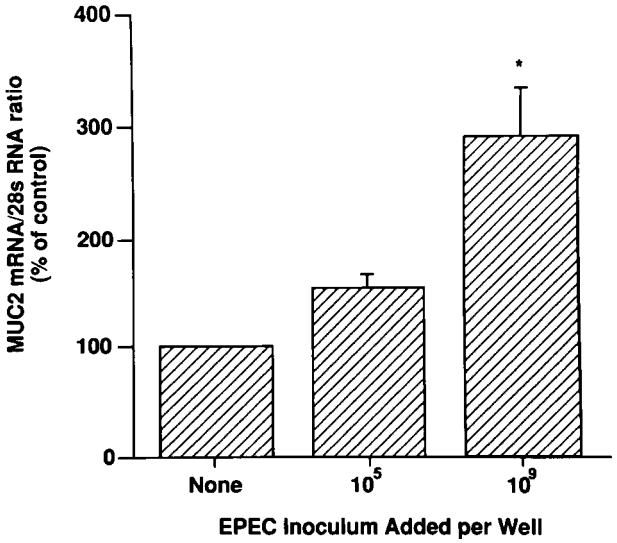

Fig. 10. Relative MUC2 mucin mRNA expression levels after EPEC strain E2348/69 incubation. Various amounts of bacteria were added to glucose-grown HT-29 cells. After a 1-h incubation, total RNA was collected, separated, and hybridized with a MUC2 cDNA probe as previously described. Results of the MUC2 signals are area integration relative to 28S RNA levels and are expressed as percent increase above control values. No differences in MUC2 mRNA expression levels were found for 10^5 EPEC/well added and controls ($P > 0.05$). Increased MUC2 mRNA expression was detected with 10^9 EPEC/well compared with both 10^5 EPEC/well and control levels (*$P < 0.05$). Results are shown as means ± SE from 3 separate experiments.

EPEC receptor on one cell line and not the other also seems improbable.

Binding to epithelial cells is the first step for many enteric pathogens to effect net fluid and electrolyte secretion, and so interruption of the enteropathogen adherence to intestinal epithelial cells could provide therapeutic benefit to the host. The process whereby EPEC inflict their characteristic attachment and effacement on epithelial cells is a multistage process, the first stage of which is characterized by an initial interaction of bacteria with the enterocyte layer. This initial attachment is thought to be mediated by the bundle-forming pilus, but other virulence factors may be involved (29). The increased intestinal mucin production elicited by probiotics could prevent the attachment of enteropathogens through steric hindrance or through the effects of greater competitive inhibition for attachment sites on mucins mimicking epithelial cell bacterial attachment sites. Specificity in the capability of mucins from different regions of the intestinal tract to inhibit EPEC in vitro adherence (22) and identification of an EHEC O157:H7 mucin receptor (32) would favor the latter explanation.

We propose that probiotic agents, such as *L. plantarum* 299v, which are able to bind to epithelial cells in vitro and colonize the intestinal tract in vivo (15), induce epithelial cells to secrete mucins that diminish enteric pathogens binding to mucosal epithelial cells. Bacterial exoproducts from *Pseudomonas aeruginosa* have been reported to induce MUC2 mucin expression (21). Lipopolysaccharide produced from the gram-

negative bacillus would not be relevant for gram-positive bacteria such as *Lactobacilli*. The effect of sterile supernatants suggests that other cell wall determinants or secreted products may be responsible for the increased intestinal mucin gene expression in epithelial cells. Whether this ability to diminish adherence is initiated through the binding of the probiotic agent to intestinal epithelial cells in vivo and/or together with a bacterial cell determinant or secreted bacterial product remains to be determined, but the effect of sterile supernatants suggests multiple possibilities are of importance.

In addition to eliciting chemokines that activate the mucosal immune responses over a period of days, intestinal epithelial cells have fast protective responses as part of their innate defenses, which include the elaboration of mucins within minutes or hours of insult. Mucins isolated from intestinal tracts of animals inhibit in vitro adherence of both animal and human EPEC strains and bind to human EHEC (7, 26, 37). HT-29 cells grown in regular glucose culture medium express significant levels of MUC2 mucin mRNA and can be induced to express high levels of MUC3 mucins by different culture conditions. In contrast, we show here that HEp-2 cells have minimal MUC2 mucin mRNA expression and no MUC3 mucin expression, and increased expression by incubation of *L. plantarum* 299v was not demonstrated. MUC2 and MUC3 mucin fractions isolated from HT-29 cell spent culture media and added exogenously to HEp-2 cells were capable of inhibiting EPEC binding. HT-29 cells are not the only intestinal epithelial cell line for which coincubation with probiotics led to diminished EPEC adherence. In previous studies by Bernet et al. (1), a *Lactobacillus acidophilus* strain was shown to inhibit EPEC binding to another intestinal cell line, Caco-2 cells. We previously showed that the Caco-2 cell line can express significant levels of MUC2, MUC3, and MUC4 mucin mRNA (13). Thus increased intestinal mucin production may also explain the previously reported effects of probiotics on Caco-2 cells.

MUC3-enriched mucin fractions inhibited EPEC binding to a greater extent than MUC2 mucins. This may be an important biological attribute of intestinal mucins, since cellular expression of MUC3 mucin is greater then MUC2 in the small intestine (12, 38). Discharges from goblet cells, which contain high levels of MUC2 mucins, are increased during inflammation of the intestinal tract and may be a secondary line of defense in addition to intestinal columnar cell mucin production. There are other possibilities for the differences in the capacities of MUC2 and MUC3 mucins to inhibit EPEC adherence. For example, HT-29 cells grown in galactose culture medium alter expression of MUC3 intestinal mucins and carbohydrate antigen expression on secreted and cell surface-associated mucins (23). Future determination of molecular interactions between EPEC and mucins may provide insight into this phenomenon.

In addition to composition, quality and quantity are also factors of intestinal mucins that may contribute to pathogen-mucin interactions (7). There are reduced numbers of goblet cells in inflammatory lesions of the bowel, such as Crohn's disease and ulcerative colitis. Mucins from inflamed colons have decreased functional capacity to bind proinflammatory molecules (6) and to inhibit bacterial binding (24). However, administration of *L. plantarum* 299v is effective in reducing enterocolitis in an animal model of intestinal inflammation (27). The dose of *L. plantarum* 299v used in our studies was similar to that used in previous studies that showed almost complete inhibition of EPEC and EHEC binding to HT-29 cells. In addition, the same dose of *L. plantarum* 299v increased expression of MUC2 mucin and MUC3 mucin mRNA after a 1-h coincubation (Fig. 9). A subagglutinating concentration of 10^5 EPEC used in our inhibition studies did not induce upregulation in the expression of MUC2 or MUC3 intestinal mucins; however, a greater inoculum was capable of this phenomenon. Infection of another intestinal adenocarcinoma cell line, T84 cells, with EPEC leads to the activation of NF-κB (34). *P. aeruginosa*-activated NF-κB has been demonstrated to bind to a κB site in the 5′-flanking region of the MUC2 gene and activates MUC2 mucin transcription in the HM3 colon epithelial cell line (21). How the *Lactobacilli* may cause increased mucin gene transcription remains to be determined.

The benefits of probiotics mediated through intestinal mucin upregulation may have broader applicability than only for bacterial enteropathogens. For instance, Yolken at al. (40) showed that intestinal mucins inhibit rotavirus replication. Because probiotics can increase expression of intestinal mucins, the reduction of both symptoms and fecal shedding after the onset of acute rotavirus gastroenteritis in children after administration of probiotic agents (9, 31, 36) may be by way of this mechanism. However, our in vitro studies did not show that EPEC could be dislodged by probiotics. Thus whether enhancement of innate defense mechanisms of intestinal epithelial cells such as mucin production is preventative or therapeutic for any specific intestinal infection remains to be determined.

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