Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro

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Background: Mucins are large complex glycoproteins that protect intestinal mucosal surfaces by limiting access of environmental matter to their epithelial cells. Several mucin genes have been described, including MUC3 that is a membrane associated mucin of the small intestine. Increased MUC3 mRNA transcription is induced by incubation of intestinal epithelial cells with a *Lactobacillus* strain known to be adherent to them.

Aims: To determine whether increased epithelial cell MUC3 mucin expression in response to *Lactobacillus* strain results in increased extracellular secretion of MUC3 mucins and the importance of epithelial cell adherence in modulation of MUC3 mucin expression.

Methods: HT29 cells grown to enhance expression of MUC3 mucins were incubated with selected *Lactobacillus* strains. Spent cell culture medium was collected for detection of secreted MUC3 mucins using dot blot immunoassay with a generated MUC3 antibody. Post-incubation HT29 cell RNA was collected for analysis of MUC3 expression by northern blot analysis using a MUC3 cDNA probe. In vitro binding studies using *Lactobacillus* strains incubated alone or coincubated with enteropathogenic *Escherichia coli* strain E2348/69 were used for adherence and inhibition of adherence studies, respectively.

Results: *Lactobacillus* strains with minimal ability to adhere to HT29 cells failed to induce upregulation of mucin gene expression. There was a direct correlation between upregulation of MUC3 mucin mRNA expression and extracellular secretion of MUC3 mucin. The same *Lactobacillus* strains that increased extracellular secretion of MUC3 mucin led to reduced adherence of enteropathogen *E. coli* E2348/69 during coincubation experiments.

Conclusion: Probiotic microbes induce MUC3 mucin transcription and translation with extracellular secretion of the MUC3 mucins. Epithelial cell adherence enhances the effects of probiotics on eukaryotic mucin expression.

We have previously shown that coincubation of *Lactobacillus plantarum* strain 299v (Lp299v) and *L. rhamnosus* strain GG (LrGG) with an enteropathogenic *Escherichia coli* (EPEC) strain E2348/69 or enterohemorrhagic *E. coli* O157:H7 strain inhibited the adherence of either of the *E. coli* strains to mucin producing intestinal epithelial cells. We also showed that incubation of Lp299v with HT29 cells could upregulate expression of MUC3 mRNA. One of the basic properties used to identify Lp299v and LrGG as potentially useful probiotics is their ability to adhere to intestinal epithelial cells.

To determine whether adherence is important to the process of mucin gene upregulation, we studied adherence of different strains of *Lactobacillus*, including a mutant strain lacking an adhesin, and measured alteration in the expression of mucin gene mRNA expression. In addition, we evaluated whether the MUC3 mucin produced was a secreted form by antibody analysis of spent cell culture medium using an antibody generated for this purpose. Finally, to show that the mucin product secreted into the cell culture medium was biologically active, we employed a bioassay to study inhibition of adherence of an enteric pathogen to the mucin producing epithelial cells.

Abbreviations: Lp299v, *Lactobacillus plantarum* strain 299v; LrGG, *L. rhamnosus* strain GG; LaDDS, *L. acidophilus* strain DDS-1; Lp adh−, *L. plantarum* strain adh−; EPEC, enteropathogenic *Escherichia coli*; CFU, colony forming units; PBS, phosphate buffered saline.
MATERIALS AND METHODS

Bacteria and growth conditions
A stock culture of EPEC strain E2348/69 (serotype O127:H6) was maintained at 4°C on trypticase soy agar slants (Becton-Dickenson Microbiology Systems, Cockeysville, Maryland, USA). Stocks of Lactobacillus strains were maintained at 4°C on MRS agar (Difco Laboratories, Detroit, Michigan, USA). Bacterial strains were kindly provided by Dr James Kaper (EPEC strain E2348/69, Center for Vaccine Development, Baltimore, Maryland, USA), and Dr Khem Shahani (L. acidophilus strain DDS-1 (LaDDS); University of Nebraska, Lincoln, Nebraska, USA).

EPEC strain E2348/69 is a member of the family of non-invasive, non-enterotoxin, diarrhoeagenic producing pathogens that produce a characteristic attachment/effacement lesion with epithelial cells. LGG (American Type Culture Collection 53103, Rockville, Maryland, USA) was originally isolated in vitro from stool specimens of healthy humans. The LaDDS strain is from a dairy source and has been reported to stimulate murine macrophage production of interleukin 1a and tumour necrosis factor α. Lp299v is a member of a genetically well defined subgroup of L. plantarum isolated from intestinal mucosa. Strains of this subgroup agglutinate *Saccharomyces cerevisiae* in a mannose sensitive manner and carry a mannose specific adhesin. The *L. plantarum* strain adh– (Lp adh–) is a spontaneous mutant of Lp299v that no longer agglutinates *S. cerevisiae* in a mannose sensitive manner. Restriction fragment length polymorphism analysis did not detect differences between Lp299v and its derivative Lp adh– (Siv Ahnre, personal communication).

Cell growth conditions
HT29 cells (American Type Culture Collection) were grown in McCoy's 5a medium to a glucose free 5 mM galactose containing McCoy's medium (Difco Laboratories, Detroit, Michigan, USA) for enhanced MUC2 expression. Cells were HT29 cells (American T ype Culture Collection) were grown in Cell growth conditions previously described.

Cell growth conditions
HT29 cells were grown to confluence in culture flasks (Falcon 3028; Becton-Dickinson, Franklin Lakes, New Jersey, USA). The same relative number of bacteria to cell contact area were added to culture flasks as were added to wells of 12 well plates for adherence assays. Specifically, 4.5×10⁹ CFU/flask (1.5×10⁹ CFU/ml) were added as the cell contact area of the culture flask is 45× greater than the cell contact area of a well of 12 well plates.

Total RNA was isolated from HT29 cells using the guanidine isothioocyanate-cesium chloride cushion ultracentrifugation technique with modifications to minimise mRNA shearing, as previously described. RNA was stored in 0.3 M sodium acetate with 2.5 volumes of ethanol at −70°C. Northern blots were formed and underwent probe hybridisation using either a cDNA probe to the tandem repeat region of MUC2 (clone SMUC41) or MUC3 (clone SIB 124; kindly provided by Drs James Gum and Young Kim), as previously described. Analysis of mucin gene signals using Phosphor screen autoradiography (Molecular Dynamics, Sunnyvale, California, USA) and area integration using ImageQuant software (version 3.3; Molecular Dynamics) relative to levels of 28s RNA were as previously described.

The reactivity and specificity of the antibody in mouse serum was determined by dot blotimmunoadsorbent assay, as described by Towbin and Gordon. Briefly, spent culture media from subconfluent HT29 cells grown to enhance MUC2 or MUC3 expression were collected separately and to purify the mucins, cell culture medium was subjected to density gradient ultracentrifugation, as described previously. Twenty μg (Lowry protein) from a mucin enriched fraction was applied as a spot onto nitrocellulose paper strips and allowed to air dry for one hour. Nitrocellulose strips were incubated with 3% bovine serum albumin (Sigma, St Louis, Missouri, USA) in 10 mM Tris-based buffer (Sigma) with 0.9% saline at pH 7.4 for one hour at 37°C. Strips were then washed with Tris saline. Mouse immune serum was added in serial dilutions for incubation overnight at 4°C. Afterwards, the strips were washed with Tris saline at room temperature to remove the unbound antibody. A 1:5000 dilution of peroxidase conjugated goat antimouse IgG (Fc’ fragment specific; Jackson ImmunoResearch, West Grove, Pennsylvania, USA) was added to the nitrocellulose
Mucin mRNA expression induced by proteolytic degradation of native mucins. Culture medium was collected separately, and to minimise ent changed to serum free medium prior to the addition of different amounts from the spent culture supernatants from cells were present. For this study, the cell culture media were added. The inhibitors included 5 mM 4-ethylmaleimide (Sigma), 2 mM phenylmethylsulphonyl fluoride (Sigma), and 0.01% sodium azide (Sigma) in a 5 mM EDTA solution (Sigma). Spent culture medium was centrifuged to remove cellular and particulate debris and then passed through a 0.02 µm filter for sterilisation. The volume of soluble supernatant was reduced by vacuum centrifugation (SpeedVac SC200; Savant Instruments, Farmingdale, New York, USA). Equivalent amounts from the spent culture supernatants from cells incubating with different Lactobacillus strains (20 µg protein) were applied as a spot onto nitrocellulose paper and air dried, and the experimentation proceeded as above. For these experiments, mouse immune serum was added at a dilution of 1:100.

Indirect immunofluorescence
To further validate that the antibody generated was to MUC3 mucin, we performed indirect immunofluorescence on normal

Figure 1: (A) Lactobacillus adherence to HT29 cells. Lactobacillus strains 10^6 colony forming unit (CFU)/well were incubated for four hours with HT29 cells grown in glucose free galactose containing cell culture medium to enhance MUC3 expression. Non-adherent bacteria were washed off (×4) and adherent bacteria were quantified by CFU determination on MRS agar plates. Lp299v, Lactobacillus plantarum strain 299v; LrGG, L rhamnosus strain GG; LaDDS, L acidophilus strain DDS-1. (B) Similar experiments were performed using the parent Lp299v strain and its adhesin negative genetic mutant Lp adh-- strain. In both, five experiments were run in triplicate and results are expressed as mean (SEM).

Figure 2: Mucin mRNA expression induced by Lactobacillus strains. Lactobacilli (4.5×10^8 colony forming units) were added to flasks in an equivalent amount as cell surface contact area of 12 well plates. After one hour of incubation, northern blots were hybridised using random-primed 32P labelled cDNA probe to the MUC3 tandem repeat. Mucin mRNA levels were quantified by area integration of phosphor screen autoradiography. Results were normalised to 28S RNA levels on agarose gels used for northern blots. Results are expressed as means (SEM) of five experiments for HT29 cells were grown in a glucose free galactose containing cell culture medium to enhance MUC3 expression. As shown in (A), MUC3 expression for cells incubated with Lactobacillus plantarum strain 299v (Lp299v) and L rhamnosus strain GG (LrGG) were increased compared with controls without bacteria added and HT29 cells with L acidophilus strain DDS-1 (LaDDS) added (*p<0.05). (B) MUC3 mRNA expression was increased for Lp299v compared with the ligand negative Lp adh-- strain (*p<0.05).
human tissue as tissue localisation in the small intestine is known. Biopsies of normal human duodenum were quick frozen in liquid nitrogen, fixed with OCT fixative, and stored at −70°C. Sections were cut 5 µm thick with a cold microtome and fixed onto microscope slides using acetate. After being warmed to room temperature and washed with PBS, the microscope slides were incubated for 30 minutes at room temperature in a 1:20 dilution of mouse anti-MUC3 mucin serum. The microscope slides were again washed with PBS, and a 1:20 dilution of fluorescein isothiocyanate conjugated to goat IgG/Fc fragment specific (Jackson ImmunoResearch) was added. The microscope slides were then incubated in darkness for 30 minutes at room temperature, washed with PBS, and examined by fluorescence microscopy. Control slides were stained with equal dilutions of PBS, fluorescein isothiocyanate alone, or preimmune mouse serum. The study protocol for obtaining a biopsy received prior approval from the Institutional Review Board of Children’s Hospital in Omaha.

Statistical analysis

Group data are expressed as means (SEM). 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 test. Analyses between two groups were determined using the two tailed unpaired Student t test.

RESULTS

Adherence of Lactobacillus strains

As shown in fig 1A, there were differences in adherence of the Lactobacillus strains to HT29 cells grown to enhance MUC3 expression. Adherence of LrGG (1.1×10⁷ (1.1×10⁷) CFU/well) was about 10x greater than Lp299v (1.6×10⁶ (1.6×10⁶) CFU/well) that was in turn about 10 times greater than LaDDS (1.3×10⁵ (86) CFU/well). There was no loss of viability of any of these Lactobacillus strains in the galactose containing cell culture medium over a similar four hour incubation period (data not shown) or during the washing procedures. Similar results were determined for binding of the Lactobacillus strains to HT29 cells grown in glucose containing cell culture medium. That is, LrGG adherence to HT29 cells (1.1×10⁷ (0.1×10⁷) CFU/well; mean (SEM)) was greater than either Lp299v (1.6×10⁶ (0.2×10⁶) CFU/well; p<0.05) or LaDDS (1.3×10⁵ (0.1×10⁵) CFU/well; p<0.05).

In separate experiments, we compared adherence of Lp299v with the mutant Lp adh− strain which has lost the ability to agglutinate Saccharomyces due to loss of production of the mannose sensitive binding ligand required for HT29 cell attachment. As shown in fig 1B, adherence of Lp299v (1.7×10⁶ (6.9×10⁵) CFU/well) to MUC3 expressing HT29 cells was about 10 times greater than the non-agglutinating Lp adh− strain (1.9×10⁵ (1.8×10⁵)) which showed minimal binding similar to the LaDDS strain in the previous set of experiments (fig 1A).

Mucin mRNA expression

Levels of MUC3 mRNA expression of HT29 cells incubated with Lactobacillus strains are shown in fig 2. Expression levels of MUC3 mRNA increased about threefold with addition of either Lp299v or LrGG compared with control cells. Both Lp299v and LrGG increased mucin gene expression levels to the same magnitude over control cells that were grown in sterile media. However, there was no difference in MUC3 mRNA expression for cells incubated with LaDDS compared with controls (p>0.05). For comparison, we also evaluated MUC2 mRNA expression as it is known to exist only as a secreted form. Similar to MUC3 mRNA expression levels, ANOVA analysis revealed that MUC2 mRNA expression levels of incubation of HT29 cells grown to express MUC2 for LrGG (304 (52)% of control) and Lp299v (227 (49)% of control) were similar and both greater than MUC2 mRNA expression by control cells incubated without bacteria or HT29 cells incubated with LaDDS (153 (27)% of control) (data not shown).

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In separate experiments, MUC3 mRNA expression levels were evaluated for the non-adhering Lp adh− compared with the parent Lp299v strain (fig 2B). The relative MUC3 mRNA expression following incubation of Lp299v (209 (31)% of control) was greater than incubation of similarly grown HT29 cells incubated with the Lp adh− strain (101 (8.5)% of control; p<0.05). There was no difference between expression levels of HT29 cells incubated with Lp adh− bacteria and cells grown under sterile conditions (p>0.05).

MUC3 mucin antiserum
To evaluate the specificity of the antiserum generated to the 9 mer MUC3 mucin backbone peptide sequence, we performed a dot blot immunooassay with mucins purified from HT29 cell spent culture medium. In fig 3, a black dot is present identifying the application site of the spent culture medium for HT29 cells grown in glucose containing medium to enhance MUC2 expression (site A) and for HT29 cells grown in glucose free galactose containing medium to enhance MUC3 mRNA expression (site B). Surrounding the application dot of site B, there is a strong reactivity to the anti-MUC3 immune serum but only minimal reaction around application site A. A minimal reaction might be expected as the HT29 cells grown in glucose containing cell culture medium conditions enhance MUC2 mucin expression but we have previously shown that there is minimal MUC3 expression present.

Indirect immunofluorescence of human small intestinal biopsy incubated with the mouse immune serum is shown in fig 4. The cross sectional view of a villus tip showed fluorescence along the surface of the epithelial cells. These results are similar to those previously published by Chang and colleagues using an antibody to the MUC3 tandem repeat region. Taken together, these results demonstrate the expected location for antigenic material derived from mucin and that the polyclonal antiserum reacts strongly with material enriched with MUC3 mucins.

MUC3 mucin extracellular expression
Anti-MUC3 serum was then used in a dot blot immunoassay using spent cell culture medium derived from HT29 cells grown in galactose containing medium incubated with different Lactobacillus strains. As shown in fig 5, a central black dot indicating the application site of the spent cell culture medium is visible in each panel. Surrounding the application spot of Lp299 and LrGG there was far greater reactivity than the blot of spent cell culture medium from HT29 cells without a probiotic added (none) or with LaDDS added.

Inhibition of enteropathogenic Escherichia coli epithelial cell adherence
We correlated the biological significance of increased MUC3 gene transcription and secreted expression of MUC3 mucins in the presence of Lactobacillus strains differing in their ability to adhere to the HT29 cells by performing an in vitro inhibition of adherence assay. For these experiments, controls were EPEC strain E2348/69 incubated with HT29 cells without Lactobacillus bacteria. As shown in fig 6A, recovery of EPEC bound to HT29 cells grown in galactose containing medium (for enhanced MUC3 expression) coincubated in the presence of Lp299v and LrGG was less than controls with EPEC alone. There was no difference in inhibition of EPEC coincubated with LaDDS compared with adherence of controls with EPEC alone for MUC3 expressing HT29 cells.

In fig 6B, the non-adherent Lp adh− strain was unable to impact the adherence of EPEC E2348/69 (8.8×10^5 CFU/well) to MUC3 producing HT29 cells. In contrast, Lp299v reduced the EPEC adherence (4.8×10^4 (3.8×10^4) CFU/well) that is similar to the effect elicited by the adhering Lactobacillus strains depicted in fig 6A.

For comparison, we also evaluated inhibition of EPEC adherence to MUC2 producing HT29 cells coincubated with different Lactobacillus strains as MUC2 is the major mucin of the colon and exists only as a secreted mucin. For HT29 cells grown in glucose containing medium (for enhanced MUC2 expression), similar results to fig 6A were determined. That is, there was decreased adherence of EPEC to HT29 cells with Lp299v (6.3×10^4 (0.7×10^4) EPEC CFU/well; p<0.005) and LrGG (1.0×10^4 (2.5×10^4) EPEC CFU/well; p>0.05) compared with controls (1.6×10^4 (3.0×10^4) EPEC CFU/well (mean (SEM)).

DISCUSSION
In the current study we confirmed that incubation of Lp299v with HT29 cells upregulated expression of MUC3 mRNA. We

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**Figure 4** Indirect immunofluorescence of human duodenum. The photomicrograph using mouse antihuman MUC3 mucin antibody as the primary antibody is shown. The cross section view of a villus from a biopsy taken from the second part of the duodenum shows fluorescence of the epithelial cell cytoplasm with increased fluorescence at the surface of the epithelial cells (arrows). Controls using preimmune serum, FITC conjugate alone, and phosphate buffered saline were negative (not shown). Magnification 40×.

**Figure 5** Immunoassay for MUC3 mucins in spent cell culture medium from Lactobacillus strains. HT29 cells were grown in glucose free galactose containing medium to enhance MUC3 expression and incubated with equivalent colony forming units of different Lactobacillus strains. Cell culture medium (10 µg protein) was blotted onto nitrocellulose. A 1:100 dilution of mouse immune serum was added. After washings, bound antibody was detected using a peroxidase colorisation reaction. A black dot in each frame is pencil marking used to indicate where medium was blotted initially. Greater immunoreactive MUC3 mucin was detected in cell culture medium incubated with Lactobacillus plantarum strain 299v (Lp299v) and L rhamnosus strain GG (LrGG) than with L acidophilus strain DDS-1 (LaDDS) and control cells without a probiotic added (none).
also showed that other Lactobacillus strains may possess this same capability but it is not a universal property of probiotic strains (fig 2). To detect MUC3 mRNA expression, a cDNA probe to the central tandem repeat region was utilised. Recently, the existence of a second MUC3 gene has been proposed.\(^5\)\(^\text{23}\) Both human MUC3 genes, now called MUC3A and MUC3B, show such significant similarity that the cDNA probe that was in these studies would recognize transcripts from both genes.\(^5\) MUC3 is a member of the membrane bound mucins along with MUC1, MUC4, and MUC12, and these various mucins can be expressed as membrane anchored, soluble, or secreted forms.\(^5\) Both membrane bound variants and splice variants coding for secreted forms\(^5\)\(^\text{23}\) have been described for MUC3 mucins. With upregulation of expression of MUC3 mRNA, the question then arises whether the mucins are cell anchored or secreted. This has biological consequences. If the upregulated mucin gene products were membrane bound, then in order for Lactobacillus strains to limit EPEC binding to intestinal epithelial cells all potential mucin binding sites for EPEC would have to be occupied by the Lactobacillus organisms. This would suggest there were common mucin binding sites for the gram positive Lactobacillus strains and the gram negative E coli. Alternatively, if the translated product of MUC3 mucin gene upregulation was a secreted mucin, then mucin EPEC interactions could occur away from the intestinal epithelial cell membrane surface and the trapped microbes could then be swept away with the peristaltic motions of the intestinal tract. As we demonstrated increased MUC3 mucins in the spent cell culture medium following probiotic incubation, the latter explanation is favoured. The rapidity of the response, which is within one hour, would match a biologically relevant time period and alteration of expression of other intestinal epithelial cell genes by non-virulent intestinal microbes has been demonstrated within a similar time frame.\(^5\)\(^\text{23}\) Rapidity of response would be expected to be an essential element for a member of the inducible innate protective response of the intestinal epithelial cell.

Traditionally, antibiotics have been administered for microbial related diseases of the intestinal tract. Their usage adds to the prevention of flare ups of chronic pouchitis following oral administration of probiotics reported by Gionchetti and colleagues\(^3\) as anaerobic bacterial concentrations correlate with the number of mucosal chronic inflammatory cells in
pouchitis. In animal models, administration of Lactobacillus reduced the severity of mucosal inflammation of colitis in interleukin 10 gene deficient mice and methotrexate induced enterocolitis in rats. The diminished inflammation correlated with reduced adherent and translocated aerobic bacteria, and Enterobacteriaceae and gram negative anaerobes, respectively. In the current study, we have confirmed that preincubation of some probiotic strains of Lactobacillus with EPEC inhibit the adherence of the enteropathogen to intestinal epithelial cells. Not all Lactobacillus strains have this capacity as the mutant strain of Lp adh− and LaDSS failed to diminish the binding of EPEC E2348/69 compared with cells grown in a sterile cell culture medium. A difference between Lp adh− and LaDSS in our assay. The difference between LrGG and Lp299v may be a result of the vigour in which washings were performed—that is, LrGG may have a greater ability to adhere to the cells compared with Lp adh− and LaDSS in our assay. The difference between LrGG and Lp299v may have about 10 times greater ability to adhere to the cell. While LrGG had the greatest ability to bind to HT29 cells, Lp299v was demonstrated to have about 10 times greater ability to adhere to the cells compared with Lp adh− and LaDSS in our assay. The difference between LrGG and Lp299v may be a result of the vigour in which washings were performed—that is, LrGG may have a greater binding strength than Lp299v. This would be in keeping with identification of LrGG in which the ability to bind to epithelial cells was a major trait expected. Alternatively, upregulation of mucin mRNA may require a threshold value of adherent organisms per cell below which no effect is elicited. This suggests adherence is necessary but does not answer the question of whether it is sufficient, but irreversible adherence may not be as biologically important to define a useful probiotic as the ability to upregulate protective epithelial cell responses such as upregulation in mucin expression as we demonstrated mucin gene expression was similar between the Lp299v and LrGG strains.

The complete sequence of MUC3 and its promoter are unknown. In contrast, for the major secreted mucin of the colon, namely MUC2, both the full length cDNA sequence and regions important for its expression have been determined which has allowed for studies on its upregulation. For instance, ligation of ASGM1, a glycolipid that functions as a bacterial receptor, results in ATP release extracellularly, and by an autocrine mechanism mediated through activation of Erk 1/2 activates MUC2 mucin transcription. Whether similar pathways are involved in MUC3 activation remains to be determined.

In summary, selected probiotic Lactobacillus species that have the ability to adhere to intestinal epithelial cells rapidly induce eukaryotic MUC3 mucin expression. The upregulated MUC3 mucin gene product is a secreted mucin that has the ability to inhibit enteric pathogen epithelial cell adherence.

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