Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC)

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Background: The colonic epithelium maintains a life long reciprocally beneficial interaction with the colonic microbiota. Disruption is associated with mucosal injury.

Aims: We hypothesised that probiotics may limit epithelial damage induced by enteroinvasive pathogens, and promote restitution.

Methods: Human intestinal epithelial cell lines (HT29/cl.19A and Caco-2) were exposed to enteroinvasive Escherichia coli (EIEC 029:NM), and/or probiotics (Streptococcus thermophilus (ST), ATCC19258, and Lactobacillus acidophilus (LA), ATCC4356). Infected cells and controls were assessed for transepithelial resistance, chloride secretory responses, alterations in cytoskeletal and tight junctional proteins, and responses to epidermal growth factor (EGF) stimulation.

Results: Exposure of cell monolayers to live ST/LA, but not to heat inactivated ST/LA, significantly limited adhesion, invasion, and physiological dysfunction induced by EIEC. Antibiotic killed ST/LA reduced adhesion somewhat but were less effective in limiting the consequences of EIEC invasion of cell monolayers. Furthermore, live ST/LA alone increased transepithelial resistance, contrasting markedly with the fall in resistance evoked by EIEC infection, which could also be blocked by live ST/LA. The effect of ST/LA on resistance was accompanied by maintenance of actin, ZO-1 and enhancement of actinin, occludin of cytoskeletal and tight junctional protein phosphorylation. ST/LA had no effect on chloride secretion by themselves but reversed the increase in basal secretion evoked by EIEC. EIEC also reduced the ability of EGF to activate its receptor, which was reversed by ST/LA.

Conclusions: Live ST/LA interact with intestinal epithelial cells to protect them from the deleterious effect of EIEC via mechanisms that include, but are not limited to, interference with pathogen adhesion and invasion. Probiotics likely also enhance the barrier function of naive epithelial cells not exposed to any pathogen.

The gastrointestinal tract harbours a complex and diverse ecosystem of microorganisms and the mechanisms by which gut immune and epithelial cells handle this constant antigenic stimulation are only beginning to be understood. Several lines of evidence have underscored the importance of a continuous cross talk between host and microbes in priming and maintaining a reciprocally beneficial relationship at various mucosal surfaces of the body. Many studies have also shown adverse consequences for the host if the normal equilibrium is disrupted.

Probiotics are defined as "living organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition." The importance of a ‘healthy’ gut microbiota has been recognised for a long time but only recently has specific attention been focused on the potential of probiotics as preventative and therapeutic agents in gastrointestinal diseases. The use of probiotics in animal models of inflammatory bowel disease and in diarrhoea of premature infants, severe burn patients, and acute and chronic colitis has shown potential beneficial effects of probiotic Escherichia coli strains, Lactobacilli, Bifidobacteria, and Saccharomyces. However, the data in this area are relatively sparse and often controversial. Probiotics have been proposed to exert their beneficial effects by maintaining a normal intestinal milieu, by stimulating the immune system, by detoxifying colonic contents, by lowering serum cholesterol levels and promoting lactose tolerance, and by producing metabolites that are essential to maintain intestinal health. However, detailed mechanism based investigations of efficacy are largely lacking.

The intestinal epithelium, the site of interface with probiotics as well as other luminal constituents, conducts several functions required for intestinal homeostasis. Among these, the ability to form a barrier to oppose permeation of solutes into the lamina propria, and to regulate fluid and electrolyte transport, have been studied extensively. Alterations in both barrier and transport functions are common sequelae of a variety of digestive disorders, and may underlie diarrhoeal symptoms. Moreover, both transport and barrier functions, and epithelial turnover and differentiation, are known to be regulated in part by signal transduction events originating from the epidermal growth factor receptor (EGFr). EGFr activation has been shown to cause redistribution of actin filaments in the apical zone of epithelia and to evoke changes in tight junctions and the cytoskeleton that are associated with an increase in transepithelial resistance (TER) and hence barrier function. Conversely, we and others have shown that infection of intestinal epithelial cell lines with invasive...
Lactobacillus acidophilus
therefore was to investigate the effects of a combined preparative pathogen, and sustain recovery of the epithelium by promoting continued EGFr signalling. The aim of this study therefore was to investigate the effects of a combined preparation of two probiotic bacteria, *Streptococcus thermophilus* (ST) and *Lactobacillus acidophilus* (LA), on intestinal epithelial cell physiology. We hoped to provide a rationale for the use of probiotics as therapeutic and preventative agents, at least in acute or chronic inflammation of the gut.

**Materials and Methods**

**Cells**

HT29/cl.19A and Caco-2 cells were cultured as previously described. Data obtained with both cell lines were qualitatively identical and have been used interchangeably in the results section. Briefly, cells were grown in McCoy's medium (Cellgro, Fisher, Philadelphia, Pennsylvania, USA) supplemented with 10% fetal calf serum, and maintained in an atmosphere of 5% CO2-95% O2 at 37°C. Cells were used between passages 5 and 25, and grown in 75 cm² flasks (Costar, Cambridge, Massachusetts, USA), or glass chamber slides (Nalge Nunc, Rochester, New York, USA). Cells grown as polarised monolayers on filters reached confluence in 7–8 days and were used consistently within 14 days from seeding, or five days post confluence.

**Bacteria**

Enteroinvasive *Escherichia coli* (EIEC O29:NM), non-invasive *S thermophilus* (ST), and *Lactobacillus acidophilus* (LA, ATCC4356) were obtained from ATCC (Rockville, Maryland, USA). Trypticase soy broth (TSB; Gibco BRL, Gaithersburg, Maryland, USA), supplemented as appropriate with 1% yeast extract (Sigma, St Louis, Missouri, USA), trypticase soy agar (TSA), or brain-heart infusion agar (Becton Dickinson, BBL, Sparks, Maryland, USA) with 5% sheep blood, or deMan-Rogosa-Sharpe broth (MRS) (Difco Labs., Detroit, Michigan, USA) were seeded with bacteria and incubated overnight at 37°C until the stationary phase was reached (table 1). ST and LA were routinely cultured under microaerophilic conditions. Subcultures of the overnight cultures in fresh medium were used to inoculate epithelial cell monolayers with bacteria in a phase of exponential growth.

**Cell Infection/Treatment and Intracellular Survival Assay**

Confluent epithelial cell monolayers were treated with serum free medium containing exponentially grown bacteria at a multiplicity of infection (MOI) of 50:1 to the apical surface. After one hour at 37°C, cells were washed and incubated in serum free medium with gentamicin (50 µg/ml) (for cells infected with invasive bacteria or uninfected controls) or in medium alone (for cells treated with probiotics) for one hour at 37°C. In control experiments, gentamicin had no effect on any of the parameters measured. Furthermore, no significant bacterial overgrowth was observed over the duration of the experiment under all conditions tested. In some experiments, cell monolayers were pretreated with ST/LA for one hour prior to infection with EIEC, as described above, and subsequent killing of extracellular bacteria with gentamicin. Some experiments were performed using probiotics that had either been killed with antibiotics (quinupristin/dalfopristin and vancomycin, 4 µg/ml, for one hour at 37°C) or heat inactivated (one hour at 100°C). Spent and filtered (0.2 µm) yolk-phospholipid, and concentrated (100x), supernatants from ST/LA cultures were also used in some experiments. Cells were then maintained at 37°C, 5% CO2 in serum and antibiotic free medium. All treated monolayers had 50% of the culture medium changed every 24 hours after infection to avoid detrimental effects from nutrient utilisation and/or variations in pH. Cell invasion and bacterial survival were checked between three and 48 hours after infection to test the reproducibility of the infection protocol. Cell lysates and supernatants from treated monolayers and controls were checked by colony forming unit (CFU) counts on TSA or MRS agar (MRS-A).

**Adherence assay**

Bacteria from log phase growth cultures were washed twice in phosphate buffered saline (PBS), pelleted by centrifugation, and suspended in 1 ml of 1x10⁶ CFU/ml in cell growth medium. Bacterial suspension (1 ml) was diluted in 1 ml of cell growth medium and the suspension overlaid on cells grown on glass slides. The treated cells were then incubated at 37°C for 1–3 hours in an atmosphere of 5% CO2-95% O2. After incubation, cells were washed with PBS, fixed in absolute methanol, Gram stained, and observed by light microscopy. Adherence of EIEC was measured by counting Gram-negative bacteria per 100 cells in 20 random microscopic fields.

**Bacterial Interference Assay**

Bacterial interference was quantified by a plate dilution method. Briefly, epithelial cell monolayers were exposed to ST/LA at 50:1 MOI for various times. Cells were then washed with warm PBS and exposed to EIEC (100:1 MOI) for one hour and subsequently tested for cell associated EIEC after hypotonic lysis using sterile distilled water supplemented with 0.1% bovine serum albumin for 30 minutes on ice. Cell associated enteropathogens were defined as adherent plus intracellular bacteria and quantified by CFU counts of diluted cell lysates on TSA. To quantify only intracellular bacteria, parallel cell monolayers were exposed to serum free medium supplemented with 50 µg/ml gentamicin for one hour at 37°C after infection with EIEC, in the presence or absence of ST/LA pretreatment, then washed and lysed as described above. EIEC invasion was expressed as a percentage of intracellular bacteria compared with total cell associated bacteria. Probiotic interference was assessed by quantifying EIEC invasion in the presence and absence of probiotic pretreatment.

### Table 1 Bacterial strains used in these studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No</th>
<th>Source</th>
<th>Gram stain</th>
<th>Growth conditions*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus acidophilus</em> (LA)</td>
<td>43S6</td>
<td>ATCC</td>
<td>Positive</td>
<td>MRS, 37°C</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> (ST)</td>
<td>BAA-250</td>
<td>ATCC</td>
<td>Positive</td>
<td>TSA-N+M, 37°C, 5% CO2</td>
</tr>
<tr>
<td>Entero invasive E coli (EIEC)</td>
<td>O29-NM</td>
<td>J Fierer UCSD</td>
<td>Negative</td>
<td>TSB-y, 37°C, 5% CO2</td>
</tr>
</tbody>
</table>

*See text for definition of abbreviations used*
Electrophysiological studies

Infected and non-infected cell monolayers were tested for chloride secretion in modified Ussing chambers at various times after infection, as described previously. Antibiotic killed (\textit{Caco}) times after infection, as described previously. 

Electrical resistance across infected and control monolayers was also measured at various times using a “chopstick" voltoh- meter (WPI, Sarasota, Florida, USA) with the inserts 

Permeability assay

Polarised cell monolayers were pretreated with probiotics for one hour, infected with \textit{EIEC} as described above, and harvested after incubation at 37°C in 5% CO₂ atmosphere for 12 hours. Fluorescein sulphonic acid (FS; Molecular Probes, Eugene, Oregon, USA; molecular weight 478 Da, 200 μg/ml) or fluorescein isothiocyanate-dextran-10S (FITC-D10; Sigma; molecular weight 10,000, 20 mg/ml) was added to the basolat- eral side. Dishes were incubated at 37°C in a 5% CO₂ incubator for one hour with gentle mixing at 15 minute intervals. After the initial incubation, at 15 minute intervals up to two hours thereafter, the FS and FITC-D10 content of apical (Ap) and basolateral (Bl) samples was measured as fluorescence intensity of 1:250 dilutions in distilled water (492 nm excitation and 515 nm emission wavelengths). Monolayer perme-

Immunoprecipitation and immunoblot analysis

Infected and uninfected cells grown on 24 mm diameter Mil- licell HA filters were harvested at various times after infection and processed as previously described.\textsuperscript{13} Proteins of interest were immunoprecipitated by overnight incubation at 4°C on a mixer with an appropriate dilution of specific antibody (anti- ZO-1, anti-occludin, and antiactinin from Santa Cruz Biotechno- logy, California, USA; anti-phosphotyrosine, anti-phospho- scine, anti-EGFr, and anti-ERK 1,2 from Transduction Laboratories, Lexington, Kentucky, USA) in cold lysis buffer\textsuperscript{41} supplemented with additional phenylmethylsulphonyl fluo- ride (20 μM). Samples were then incubated with protein A agarose at 4°C for one hour with constant mixing. After wash-

Results

Effects of probiotic colonisation on intestinal epithelial cell barrier function

To investigate any direct effects of probiotics on our cell line models, we selected ST and LA, and exposed HT29/cl.19A and Caco-2 cells to each individual strain and to a combination of the two (ST/LA). The two probiotics were not toxic to the cell monolayers, as shown by viability measurements (trypan blue exclusion assay, and HO342 and rhodamine fluorescence staining) following incubation with various MOI (10–5000:1) of bacteria for up to 48 hours compared with untreated controls (data not shown). Furthermore, no significant bacte- rial overgrowth was observed over the duration of the experi- ment under all conditions tested. Moreover, neither strain altered the basal chloride secretory function of intestinal epi- thelial cells (not shown). In contrast, the combination of the two probiotics caused a small but significant increase in TER (fig 1A). This effect on TER was also corroborated by the effect of ST/LA on permeability of infected monolayers to the small molecular tracer FITC (table 2). However, neither ST or LA, nor both strains in combination, altered permeability to a 10 kDa tracer (table 2). Spent medium from ST or LA cultures, ST/LA killed with antibiotics (fig 1B), and heat inactivated probiotics failed to increase TER or decrease permeability to FITC (not shown). We conclude that live probiotics improve epithelial barrier properties. This is a potential mechanism contributing to their beneficial effect in vivo.
Pretreatment with probiotics prevents EIEC induced decrease in TER

We and others have previously shown that a prominent effect of invasive pathogens is to reduce TER.\(^7\) \(^8\) \(^9\) \(^10\) \(^11\) Because probiotics alone improved epithelial barrier function, we next explored whether they might also abrogate the deleterious effects of pathogens on this parameter. HT29/cl.19A (fig 2A) or Caco-2 (not shown) cells were exposed to medium alone, to ST/LA and EIEC, or to EIEC or ST/LA alone. Probiotic treatment consisted of live bacteria, antibiotic killed, or heat inactivated preparations. EIEC caused a decline in TER beginning at 6–12 hours after infection compared with uninfected control monolayers whereas pretreatment with live probiotics prevented this effect. The EIEC evoked fall in TER was also seen even when probiotics were added one hour after EIEC (data not shown). However, if antibiotic killed or heat inactivated probiotics, or ST/LA spent medium were used to perform experiments, these experiments were performed three times in triplicate.

<table>
<thead>
<tr>
<th>Bacteria/probe</th>
<th>HT29/cl.19A</th>
<th>Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>395 (88)</td>
<td>532 (110)</td>
</tr>
<tr>
<td>FITC-D10</td>
<td>98 (22)</td>
<td>126 (35)</td>
</tr>
<tr>
<td>EIEC</td>
<td>276 (33)</td>
<td>321 (66)*</td>
</tr>
<tr>
<td>FITC-D10</td>
<td>82 (15)</td>
<td>89 (34)</td>
</tr>
<tr>
<td>FS</td>
<td>245 (14)*</td>
<td>299 (30)*</td>
</tr>
<tr>
<td>ST/LA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FITC-D10</td>
<td>80 (9)</td>
<td>91 (21)</td>
</tr>
<tr>
<td>EIEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FITC-D10</td>
<td>78 (1)</td>
<td>84 (9)</td>
</tr>
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</table>

Table 2 Permeability of HT29/cl.19A and Caco-2 cells after treatment with EIEC and ST/LA in vitro

Pretreatment with probiotics prevents EIEC induced increases in epithelial permeability to small molecules and macromolecules

The effects of EIEC, with or without probiotics, on TER measurements was suggestive of corresponding effects on epithelial permeability. To examine this directly, we measured the flux of two fluorescent probes of differing size across infected and control monolayers. After infection with EIEC, the permeability of HT29/cl.19A and Caco-2 cells, expressed as clearance of the two fluorescent probes tested, was markedly increased and the alteration was proportionally greater for the smaller probe (table 2). Moreover, pretreatment only with live probiotics prevented the increase in permeability induced by EIEC infection for up to 24 hours after EIEC exposure in both cell lines whereas antibiotic killed or heat inactivated ST/LA failed to exert this effect. Furthermore, simultaneous inoculation of ST/LA and EIEC at equivalent MOI, or exposure of the cells to probiotics one hour after EIEC infection, also failed to prevent the increase in FS or FITC-D10 permeability (table 2). Moreover, even at higher MOI, simultaneous addition of probiotics failed to prevent permeability changes induced by EIEC despite the fact that this treatment reduced EIEC invasion (see below).
Pretreatment with probiotics limits colonisation of EIEC in epithelial cell monolayers

We next explored whether our probiotic preparation altered the interaction of EIEC with the epithelium. As shown in table 3, the number of adhered EIEC was significantly reduced when monolayers were preincubated with equal numbers of each of ST and LA. Moreover, if the relative numbers of the probiotic strains were increased while the EIEC inoculum remained constant at 50:1, adhesion of EIEC was essentially abolished, particularly at later time points. Simultaneous addition of probiotics and EIEC was also able to inhibit adhesion of the latter bacteria, although only with an increased number of probiotics and to a lesser extent than seen with preincubation. In contrast, if probiotics were added one hour after EIEC infection, no significant effect on EIEC adhesion was seen. Interestingly, antibiotic killed ST/LA maintained the ability to inhibit EIEC adhesion, whereas heat inactivated probiotics or spent medium did not (table 3).

Table 3: Effect of ST/LA on adhesion of EIEC to Caco-2 cell monolayers

<table>
<thead>
<tr>
<th>Time after EIEC addition (h)</th>
<th>None</th>
<th>Live ST/LA 1 h prior to EIEC</th>
<th>Live ST/LA simultaneous with EIEC</th>
<th>Live ST/LA 1 h after EIEC</th>
<th>ST/LA (antibiotic killed) 1 h prior to EIEC</th>
<th>ST/LA (antibiotic killed) simultaneous with EIEC</th>
<th>ST/LA (heat inactivated) 1 h prior to EIEC</th>
<th>ST/LA (heat inactivated) simultaneous with EIEC</th>
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<tbody>
<tr>
<td></td>
<td>N/A</td>
<td>2:1</td>
<td>2:1</td>
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<tr>
<td>Ratio of MOI‡ ST/LA:EIEC</td>
<td>24 (3)</td>
<td>6 (2)</td>
<td>19 (4)</td>
<td>22 (5)</td>
<td>10 (2)</td>
<td>18 (2)</td>
<td>5 (1)</td>
<td>21 (2)</td>
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<td>27 (1)</td>
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</table>

We next explored whether our probiotic preparation altered the interaction of EIEC with the epithelium. As shown in table 3, the number of adhered EIEC was significantly reduced when monolayers were preincubated with equal numbers of each of ST and LA. Moreover, if the relative numbers of the probiotic strains were increased while the EIEC inoculum remained constant at 50:1, adhesion of EIEC was essentially abolished, particularly at later time points. Simultaneous addition of probiotics and EIEC was also able to inhibit adhesion of the latter bacteria, although only with an increased number of probiotics and to a lesser extent than seen with preincubation. In contrast, if probiotics were added one hour after EIEC infection, no significant effect on EIEC adhesion was seen. Interestingly, antibiotic killed ST/LA maintained the ability to inhibit EIEC adhesion, whereas heat inactivated probiotics or spent medium did not (table 3).

Figure 3A and 3B depict EIEC invasion of intestinal epithelial cells under the various conditions tested. Gentamicin was added after one hour of EIEC infection to eliminate extracellular bacteria. These data paralleled, in part, those presented in fig 3 of previous studies. As shown in fig 3B, the number of adhered EIEC was significantly reduced when monolayers were preincubated with equal numbers of each of ST and LA. Moreover, if the relative numbers of the probiotic strains were increased while the EIEC inoculum remained constant at 50:1, adhesion of EIEC was essentially abolished, particularly at later time points. Simultaneous addition of probiotics and EIEC was also able to inhibit adhesion of the latter bacteria, although only with an increased number of probiotics and to a lesser extent than seen with preincubation. In contrast, if probiotics were added one hour after EIEC infection, no significant effect on EIEC adhesion was seen. Interestingly, antibiotic killed ST/LA maintained the ability to inhibit EIEC adhesion, whereas heat inactivated probiotics or spent medium did not (table 3).
EIEC induced a significant increase in basal $I_{sc}$ compared with controls. Pretreatment with probiotics significantly reduced enteroinvasive Escherichia coli (EIEC) invasion of infected Caco-2 cell monolayers. (A) Caco-2 cells were exposed to EIEC alone, Streptococcus thermophilus and Lactobacillus acidophilus followed by EIEC (ST/LA+EIEC), ST/LA and EIEC simultaneously (ST/LA/EIEC), or EIEC followed by ST/LA (EIEC+ST/LA), and treated with gentamicin one hour after EIEC infection to kill extracellular bacteria. Living, antibiotic killed, and EIEC simultaneously (ST/LA/EIEC), or EIEC followed by ST/LA (EIEC+ST/LA), and treated with gentamicin one hour after EIEC infection to kill extracellular bacteria. Living, antibiotic killed, and heat inactivated ST and LA were tested in these experiments. (B) (pretreatment) and (C) (simultaneous treatment) summarise invasion data obtained at various ST/LA multiplicity of infection (MOI) (EIEC 50:1 MOI). (D) Results of invasion in cell monolayers pretreated with inactivated probiotics plus their spent medium (SM) or spent medium alone. Data are means (SEM), $n=6$. *p<0.05, **p<0.01, ***p<0.001 compared with cells infected with EIEC in the absence of probiotics (by ANOVA).

Pretreatment with probiotics reduces enteroinvasive Escherichia coli (EIEC) invasion of infected Caco-2 cell monolayers. Pretreatment with ST/LA blocked the ability of EIEC to potentiate secretion evoked by galanin (fig 7). Again, simultaneous exposure to EIEC and probiotics or addition of probiotics after EIEC failed to reverse the potentiated secretion (fig 7), independent of the size of the probiotic inoculum. These findings correlate to some degree to the results of adhesion and invasion experiments, although reduced adhesion evoked by simultaneous addition of probiotics and EIEC, especially at higher ST/LA MOIs, is not sufficient to prevent pathogen evoked hypersecretion. On the other hand, ST/LA spent medium was able to substantially prevent EIEC induced hypersecretory responses to galanin. As before, our data underscore the fact that both live probiotics and their secreted products can impact epithelial responses to pathogens but only the former can exert the full spectrum of preventative effects.

Pretreatment with probiotics prevents EGF receptor inactivation induced by EIEC infection

EIEC infection can prevent activation and/or increased degradation of the EGF receptor. Given the critical role of EGF and the EGFr in many aspects of gastrointestinal physiology and epithelial repair, we hypothesised that the beneficial effect of probiotics might extend to their ability to restore EGFr signalling in epithelial cells infected with EIEC. In fact, when cells were pretreated with probiotics, infected with EIEC, then...
Dephosphorylation in enteroinvasive Escherichia coli (EIEC) alters phosphorylation of the tight junction proteins occludin and ZO-1. HT29/Cl.19A cell monolayers were exposed to medium, Streptococcus thermophilus and Lactobacillus acidophilus (ST/LA) [multiplicity of infection=50:1], EIEC, or ST/LA followed by EIEC. Both living and antibiotic killed (†) ST and LA were tested in these experiments. Cells were harvested at 12 hours after infection. Whole cell lysates were immunoprecipitated with specific antibodies against occludin (A) or ZO-1 (B), then blotted with the same antibody or with an antiphosphoserine (A) or antiphosphotyrosine (B) antibody. The upper panels are representative blots whereas the lower panels show means (SEM) for densitometric analyses, n=6. ***p<0.001 compared with uninfected controls (by ANOVA).

Pretreatment with probiotics limits enteroinvasive Escherichia coli (EIEC) induced increases in chloride secretion. HT29/Cl.19A cells were exposed to medium, EIEC, Streptococcus thermophilus and Lactobacillus acidophilus (ST/LA) alone on the apical side. Preparations of living or antibiotic killed (†) ST/LA, or spent medium (SM) from ST and LA cultures were tested in these experiments. Short circuit current (Isc), an indicator of chloride secretion, was measured at the times indicated. Data are means (SEM), n=6. ***p<0.001 compared with uninfected controls (by ANOVA).

Mitogen activated protein kinases are downstream effectors of the EGFr. Both EIEC and live probiotics induced early phosphorylation of the ERK,1,2 isoform of these kinases (fig 9). Antibiotic killed (fig 9) or heat inactivated (not shown) ST/LA alone were unable to activate ERK1,2. We also tested whether ERK 1 and 2 were activated under the various conditions studied following EGF stimulation. Pretreatment with live probiotics reversed the inhibition of ERK 1 and 2 activation induced by EIEC infection in a significant fashion (fig 9). There was no significant change in total ERK levels under any condition. Antibiotic killed (fig 9) or heat inactivated (not shown) probiotics failed to reverse the effects of EIEC infection on EGF induced ERK1,2 phosphorylation (fig 9). These data further corroborate that probiotics likely have myriad protective effects in the intestinal epithelium.
Figure 7  Pretreatment with probiotics prevents galanin stimulated chloride secretory responses in enteroinvasive Escherichia coli (EIEC) infected HT29/cl.19A cell monolayers. HT29/cl.19A cell monolayers were exposed to medium alone, Streptococcus thermophilus and Lactobacillus acidophilus (ST/LA) alone, EIEC alone, ST/LA followed by EIEC (ST/LA/EIEC), ST/LA and EIEC simultaneously (ST/LA/EIEC), or EIEC followed by ST/LA (EIEC+ST/LA), or spent medium (SM) from ST and LA cultures with or without EIEC. Preparations of living or antibiotic killed (†) ST/LA were used. Monolayers were mounted in Ussing chambers at the times indicated and chloride secretory responses to galanin (100 nM) assessed as maximal increases in short circuit current (ΔIsc). *p<0.05, ***p<0.001 compared with uninfected controls (by ANOVA).

DISCUSSION
The role of commensal and probiotic bacteria in the physiology of the gastrointestinal tract is incompletely understood. In particular, the mechanisms underlying the benefits derived from biotherapy with probiotics have not been sufficiently elucidated, with available data largely fragmentary and/or anecdotal. It is known that stimulation by commensal bacterial antigens is crucial for the normal development of the mucosal immune system and maintenance of tolerance. Moreover, probiotics have been shown to attenuate or abolish tumour necrosis factor α stimulated interleukin 8 production in intestinal epithelial cells. The studies described here show in addition that, by diverse mechanisms, living probiotics are able to prevent or counteract the full spectrum of epithelial dysfunction induced by an enteroinvasive pathogen. In addition, probiotics alone, in the absence of EIEC infection, had effects that are arguably beneficial. Thus addition of live probiotics to naïve epithelial monolayers resulted in a progressive increase in their TER and an accompanying decrease in their permeability to a low molecular weight tracer.

Our findings demonstrate the ability of live probiotic bacteria to interfere with enteric pathogens through diverse mechanisms. Our data concur with recent work from other laboratories in showing an important, but not exclusive, role for probiotic induced interruption of the early interactions of pathogens and host cells. Mack et al showed that part of the beneficial effect of L. plantarum and L. ramosus was mediated by induction of mucus genes in intestinal epithelial cells, thus preventing adherence of enteropathogenic E. coli. We do not know yet if S. thermophilus or L. acidophilus have similar effects in our models. However, only pretreatment with probiotics prevented invasion of EIEC into epithelial cells and some, but not all, aspects of epithelial dysfunction associated with invasion, whereas simultaneous exposure to both probiotic and pathogenic bacteria had lesser preventative effects, especially at equivalent MOIs.

In our study, probiotic exposure following EIEC infection of epithelial monolayers had essentially no ability to prevent colonisation and invasion, or functional sequelae. Our findings are mirrored in the reported need to administer large doses of...
probiotics in vivo when attempting to control or attenuate ongoing gastrointestinal infections or inflammation. Specifically, Madsen et al showed that the use of a single probiotic strain alone was beneficial in reducing disease in IL-10 deficient mice only at high densities. Moreover, work in our laboratory suggests that different probiotics produce differing levels of bactericidal proteins with various degrees of efficacy against enteric pathogens, as well as autoinducers that can condition the formation of complex biofilms above the epithelial surface, conceivably thus modulating mucosal responses to the luminal environment (Resta-Lenert et al, unpublished observations). These findings emphasise the importance of investigating a diversity of host-microorganism interactions when profiling probiotics. Probiotics which display different characteristic phenotypes may be valuable as therapeutic tools under different pathophysiological conditions.

While either pretreatment or simultaneous inoculation (at appropriate MOIs) with live probiotics could limit EIEC invasion, under different pathophysiological conditions. In previous studies, products or host factors, are able to dysregulate secretory consequences of bacterial invasion, either in terms of bacterial barrier functions. Moreover, the findings imply that the medium also restored normal transport function. These data with live ST/LA was able to block the effect of EIEC on chloride secretion, after injury in several gastrointestinal pathological conditions


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